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From The Arctic Research Laboratory, Point Barrow, Alaska.

The Respiratory Response to Acute Exercise of Eskimoes and White.

By

H. ERIKSON.¹

Received 29 May 1957.

Earlier investigations by BENEDICT and CATHCART (1913), HILL and collaborators (1923—25), SIMONSON (1926), LIEBENOW (1928), HEBESTREIT (1929), MARGARIA, EDWARDS and DILL (1933) and BERG (1947) have shown that the respiratory gaseous exchange (*i. e.* oxygen uptake, carbon dioxide output and pulmonary ventilation) after muscular exercise remains increased for a period of time, which depends on the intensity and duration of the exercise as well as the cardiopulmonary function of the subject.

An earlier study by the author (1957) reports on the respiratory gaseous exchange during the recovery after a 70 yard run by trained and untrained subjects, and the findings are compared with the results of the investigations cited above. In accordance with the results reported by BERG, the time required for elimination of excess carbon dioxide, *i. e.* CO₂ recovery time, was found to show marked inter-individual differences while showing the least intra-individual variations.

The present investigation was undertaken to compare the respiratory response to exercise of eskimoes and white people living in the same area. It was carried out at The Arctic Research Laboratory, Point Barrow, Alaska in 1948—49.

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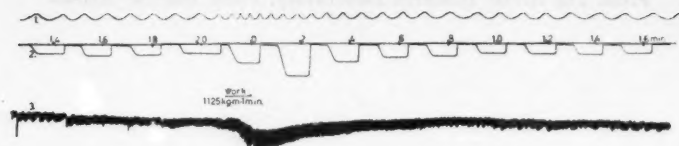


Fig. 1. Section of spirometer tracing from experiment on subject no. 2.

1. Ventilation.
2. Carbon dioxide output.
3. Respiratory difference curve.

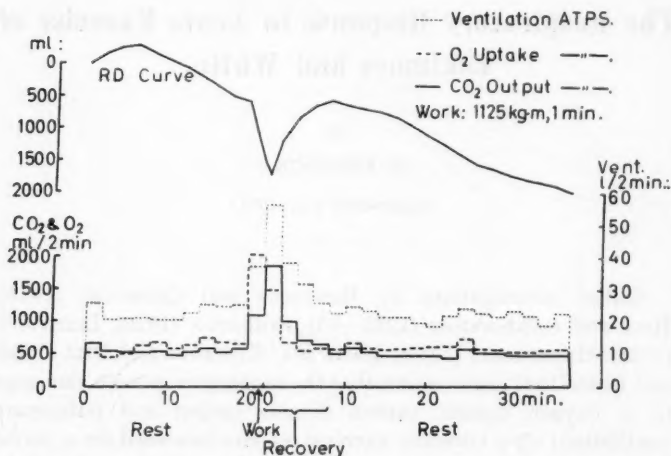


Fig. 2. Diagram of the respiratory gaseous exchange during rest, work and recovery, from experiment on subject no. 2.

Methods.

The respiratory gaseous exchange was determined by means of a spirometer described by ERIKSON, SCHOLANDER and IRVING (1951), which records a continuous respiratory difference curve (SCHOLANDER 1940), a continuous pulmonary ventilation tracing, and which gives the carbon dioxide for 2 minute periods.

The exercise was performed on a bicycle ergometer with a mechanical brake. Instead of a bicycle seat, a comfortable chair was used and the pedals were placed in front of, not below, the subject. In this way, the possibility of utilizing the weight of the body and the lower extremities for pedalling was eliminated.

An example of a spirometer tracing is given in fig. 1 and the values of the respiratory gaseous exchange thus determined in fig. 2.

Material.

Twelve male eskimoos, from 16 to 24 years old, from Barrow village and ten white males, from 18 to 23 years old from The Arctic Test Station, U. S. Navy, Point Barrow, volunteered as experimental subjects. None of the eskimoos had ever seen a bicycle before, while all the subjects from Arctic Test Station were used to bicycling.

Of the twelve eskimoos, two did not turn up for repeated experiments, leaving ten eskimoos and ten white for consideration.

On each of the ten eskimoos from 2 to 6 successful experiments were obtained, and on each of the ten white from 3 to 4 experiments.

Procedure.

The experiments were conducted at different times of the day. The subjects were instructed to avoid exercise and meals the last 1—2 hours before the experiments.

Upon arrival in the laboratory, the subjects were seated in the ergometer chair where they rested for 15—20 minutes before being connected with the spirometer. The respiratory gaseous exchange was then recorded for 20 to 30 minutes during rest, followed by the performance of approximately 1,125 kg-m work on the ergometer in the course of 1 minute, then followed by 30 to 40 minutes of recovery and rest.

Results.

The resting respiratory exchange.

The oxygen uptake, the carbon dioxide output and the pulmonary ventilation were found to decrease gradually the first 6 to 20 minutes after connection of the subject to the spirometer, similar to the findings described in a previous paper (ERIKSON 1957). The resting values were therefore in each experiment determined as the average value of a 10 to 20 minutes' rest interval when a constant level was reached.

The physical characteristics, mean oxygen uptake and carbon dioxide output, pulmonary ventilation and metabolic rate (calculated according to DuBois and DuBois 1916, and Lusk 1928, and expressed in per cent of predicted basal metabolism) of all subjects are presented in table I.

The gas volumes were measured at temperatures between 16 and 20° C.

Table I.
Respiratory exchange of eskimoese and white during rest.

Sub- ject ¹	Age years	Height cm	Weight kg	Vital Capac- ity l ²	No. of Ex- peri- ments	O ₂ Up- take ml/ min. ³	CO ₂ Out- put ml/ min. ³	Pulm. Venti- lation l/min. ³	Meta- bolic Rate %
1	23	166	73	5.6	6	356	292	10.7	129
2	20	165	60	—	2	336	274	12.1	133
3	18	160	67	4.4	4	337	296	11.3	128
4	22	—	—	4.8	4	329	293	11.5	—
5	16	163	57	3.5	6	321	263	9.4	122
6	21	168	69	4.5	2	323	272	9.4	120
7	19	168	74	4.7	6	367	304	10.1	127
8	16	173	60	4.5	5	291	231	10.4	102
9	20	—	—	4.2	2	306	250	11.7	—
10	24	170	68	4.6	2	271	245	11.3	102
Mean 1—10.	19.9	166.6	66.0	4.5		323.7	272.0	10.79	120
11	21	191	79	4.1	3	328	250	10.1	102
12	23	171	66	5.0	3	303	260	9.8	114
13	18	180	70	4.8	4	298	261	9.6	102
14	21	182	92	5.3	4	373	311	12.3	114
15	21	178	66	4.6	3	305	246	10.3	112
16	21	170	65	3.9	4	290	253	11.9	111
17	18	185	75	5.2	4	326	267	10.0	105
18	21	179	68	5.2	3	297	238	9.7	106
19	23	184	77	5.7	4	252	210	7.4	83
20	22	160	57	4.8	3	214	176	5.3	89
Mean 11—20.	20.9	178.0	71.5	4.9		298.6	247.2	9.64	104

¹ Subjects 1—10 are eskimoese. Subjects 11—20 are white.

² Corrected to BTPS.

³ ATPS.

The data given in table I show that the eskimoese were smaller, but had higher metabolic rate during rest than the white; the mean oxygen uptake being 324 and 299 ml/min. respectively at R. Q.'s of 0.84 and 0.83. The difference in resting metabolic rate is larger if the differences in body weight and height are considered.

The pulmonary ventilation per 100 ml oxygen uptake was 3.3 l for the eskimoese and 3.2 l for the white.

The respiratory rates of the eskimoese varied from 11 to 27 respirations per minute, and of the white from 5 to 23, the mean values being respectively 19 and 14.

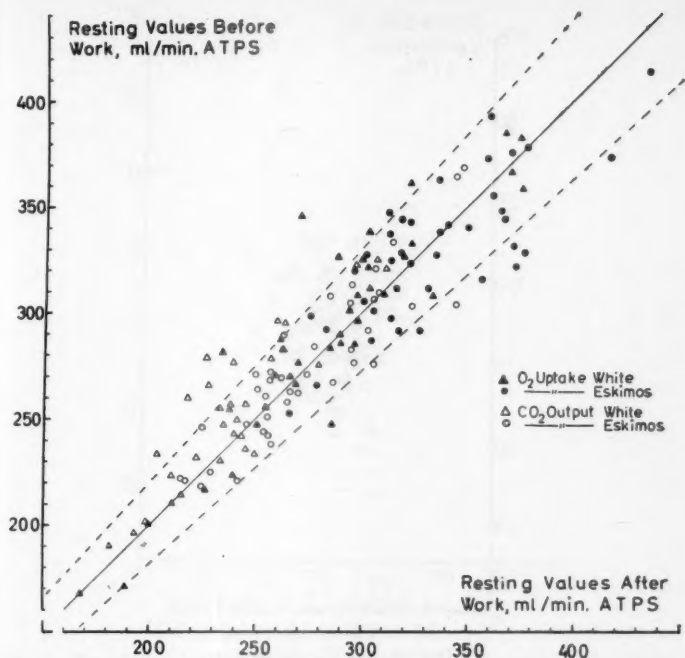


Fig. 3. The relation between resting values of oxygen uptake and carbon dioxide output before and after work.

The drawn lines represent resting values before work equal to resting values after work. The dotted lines ± 10 per cent.

The effect of the exercise.

During the work period a metronome set at 120 beats per minute was placed in front of the subject, who was instructed to make one half turn of the bicycle pedal for each beat. The one minute of work thus resulted in about 60 revolutions of the pedal with 132 revolutions of the bicycle wheel. The wheel had a circumference of 188 cm, on which a breaking force of 4.5 kg was used, resulting in a work of approximately 1,125 kg-m.

After the exercise, the oxygen uptake, carbon dioxide output and pulmonary ventilation were increased for 6 to 18 minutes, but then resting values were again obtained.

The relation between pre- and post-exercise resting values of oxygen uptake and carbon dioxide output is shown on fig. 3.

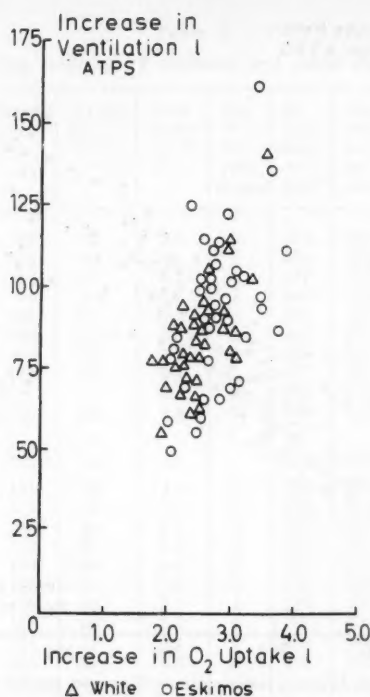


Fig. 4. The relation between increase in oxygen uptake and ventilation in 74 experiments on 20 subjects.

The increase in the respiratory gaseous exchange due to the exercise was determined by subtracting the mean of the pre- and post-exercise resting values from the total exchange during the exercise and recovery.

The relation between the increase in pulmonary ventilation and oxygen uptake is shown in fig. 4, and between increase in ventilation and carbon dioxide output in fig. 5.

The increase in oxygen uptake, carbon dioxide output and the recovery times for oxygen uptake and carbon dioxide output (*i. e.* the number of minutes from completion of the work until resting values are regained) are shown in table II.

In these experiments the eskimo subjects were found to increase their oxygen uptake with 1.7 to 2.6 ml (at S. T. P. D.) per kg-m of external work with a mean value of 2.2 ml oxygen per kg-m.

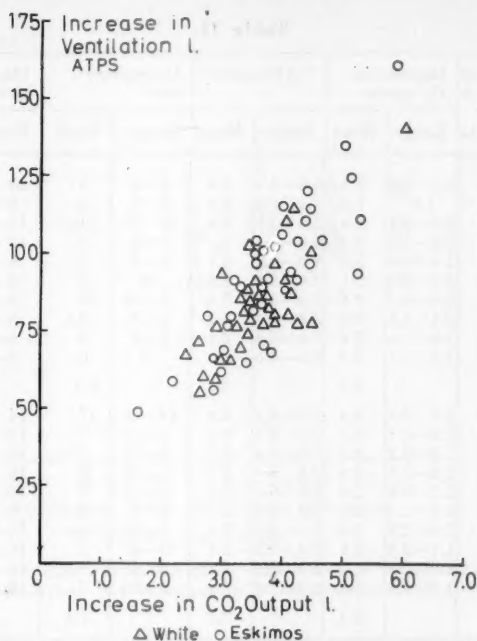


Fig. 5. The relation between increase in carbon dioxide output and ventilation in 74 experiments on 20 subjects.

The white subjects used from 1.7 to 2.6 ml oxygen in addition to the resting uptake per kg-m, with a mean value of 2.0 ml oxygen per kg-m.

If we assume a R. Q. of 1.0 for the working metabolism, the caloric value of each liter oxygen is 5,047 Calories (Lusk 1928). The net work efficiency of both eskimoes and white, varied from 27.5 to 18.0 per cent, the mean values being respectively 23.8 and 26.1 per cent. The R. Q. obtained in this type of work cannot be assumed to reflect the metabolism, but the caloric values of oxygen is relatively independent of the R. Q., increasing with only 5 per cent at a change from 0.80 to 1.00.

The recovery time for oxygen varied considerably in repeated experiments on the same subject, even when the increase in oxygen uptake was fairly constant (especially subjects 12, 14 and 20 in table II), while the recovery time for carbon dioxide was rather

Table II.

Subject	No. of experiments	Increase in O ₂ -uptake		"CO ₂ -excess"		O ₂ -recovery time		CO ₂ -recovery time	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
1....	6	2.1-3.5	2.8	3.8-4.8	4.3	2-6	5	10-14	12
2....	2	1.9	1.9	1.4-2.5	2.0	2-6	4	6-8	7
3....	4	2.6-3.4	2.9	2.9-3.7	3.3	10-16	12.5	10-14	11
4....	4	2.0-2.5	2.3	2.9-4.0	3.5	2-6	3	10-14	13
5....	6	1.9-2.3	2.2	2.0-3.2	2.7	2-4	2	6-8	7
6....	2	2.2-2.8	2.5	2.6-3.2	2.9	4	4	8-10	9
7....	6	2.3-3.2	2.7	3.2-4.0	3.5	8-16	10	8-12	10
8....	5	2.4-3.3	2.8	3.6-5.3	4.2	2-8	3.5	8-12	9.5
9....	2	1.9-2.3	2.1	2.8-2.9	2.9	2-4	3	8-10	9
10....	2	2.4-2.7	2.5	3.3-4.0	3.7	6	6	8-12	10
Mean			2.5		3.3		5.3		9.75
11....	3	2.3-3.3	2.9	3.8-5.4	4.4	16-18	17	14-16	15
12....	3	2.2-2.7	2.5	2.8-4.1	3.5	4-10	7	10-12	11
13....	4	1.9-2.7	2.3	3.0-3.8	3.3	4-12	7.5	10-14	12
14....	4	2.0-2.6	2.3	2.7-3.7	3.1	4-18	8	10-18	13.5
15....	3	2.1-2.6	2.2	2.4-3.7	3.1	8-12	9	10-12	11
16....	4	1.7-2.2	2.0	2.2-3.1	2.7	2-4	3.5	8-10	8.5
17....	4	1.9-2.6	2.3	2.4-3.5	3.1	8-14	10.5	10-14	11.5
18....	3	1.7-2.8	2.2	2.4-3.3	2.8	2-8	5	10-12	11
19....	4	1.9-2.2	2.1	3.0-3.7	3.3	2-4	3.5	10-12	10.5
20....	3	1.6-2.2	1.9	2.9-3.4	3.1	2-16	7	10-12	11
Mean			2.3		3.2		7.8		11.5

Subjects 1 to 10 are eskimoës. Subjects 11 to 20 are white.

Oxygen debt and carbon dioxide excess are given in liters at S. T. P. D.

Oxygen and carbon dioxide recovery times are given in minutes.

constant for each subject, except subject 14. The recovery times for carbon dioxide were considerably longer than for oxygen.

The increase in carbon dioxide output due to the exercise varied considerably, but these variations did not result in a corresponding change in the recovery times for carbon dioxide (see fig. 6).

The recovery times of the eskimoës were shorter than for the white in spite of a slightly lower working efficiency.

Discussion.

Under resting conditions eskimoës were found to have a higher metabolic rate than the white, the mean values being respectively 120 and 104 per cent of the predicted basal metabolism. But the white subjects were obviously more relaxed than the eskimoës. The

Increase in
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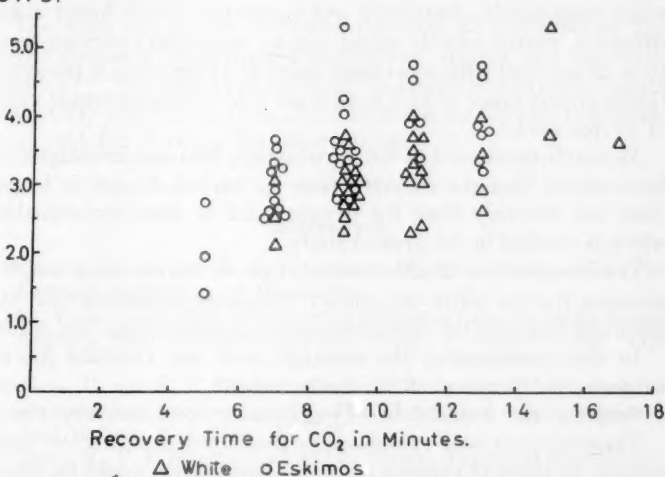


Fig. 6. The relation between increase in carbon dioxide output and recovery time for carbon dioxide output in 74 experiments on 20 subjects.

experiments on subjects 19 and 20 were performed in the mornings and these two had not eaten breakfast. Even so, their metabolic rate was exceptionally low (83 and 89 per cent).

Furthermore, in a previous study by the author (ERIKSON 1957) the resting (not basal) metabolism of 13 American male students was determined with exactly the same method and found to vary from 103 to 128 per cent of the predicted metabolism, with a mean value of 113 per cent. The apparent higher metabolism of the eskimoes in this limited material is therefore in the author's opinion, not significant.

The relations between metabolism and pulmonary ventilation in the two groups were practically identical.

The increase in oxygen uptake, carbon dioxide output and pulmonary ventilation due to the exercise was almost identical in the two groups. Since the eskimoes previously never had used a bicycle, and the white subjects all were used to bicycling, this indicates that with the type of bicycle ergometer used in these experiments, the net work efficiency appears to be independent of whether the subjects are used to bicycling or not, as was found by BENEDICT and CATHCART (1913).

The work efficiency was found to vary from 18 to 27.5 per cent with an average value of 24 and 26 per cent for the eskimoes and white respectively. BENEDICT and CATHCART (1913) found a net efficiency during bicycle riding (on an ergometer) varying from 10 to 25 per cent with an average value of 21 per cent. KARPOVICH (1953) gives a range of 14.5 to 24.9 per cent, with an average value of 21 per cent.

As briefly mentioned in the introduction, previous investigations have shown that the recovery time for carbon dioxide is longer than the recovery time for oxygen, and is more reproducible, which is verified in the present study.

The mean carbon dioxide recovery time for the eskimoes was 9.8 minutes, for the white subjects 11.5 minutes, indicating that the eskimoes had a better cardiorespiratory function than the white.

In this investigation the external work was constant for all subjects, independent of the body weight.

Subjects no. 2 and 5 had exceptionally short recovery times.

These subjects were exceptionally good hunters with a low body weight. In types of exercise where the main stress would be lifting of the body weight of the subject, their performance would be even more striking.

Summary.

The respiratory gaseous exchange of 10 Alaskan male eskimoes and 10 white male subjects during rest and during and after a work performance of approximately 1,125 kg-m in the course of 1 minute on a bicycle ergometer was determined.

During rest the only difference between the two groups of subjects was that the eskimoes had a higher resting metabolism. The difference in this limited material might be accidental, or can also be due to the fact that the eskimoes under these circumstances were more tense than the white.

The work efficiency of the eskimoes, who never had bicycled before, was almost the same as of the white, who all were used to bicycling.

The eskimoes, who were shorter and lighter than the white subjects had a shorter recovery time indicating a better cardio-respiratory function. For types of exercise which are a function of the body weight, the difference would have been even more pronounced.

Acknowledgements.

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Supersensitivity of Salivary Glands of Rabbits.

By

I. NORDENFELT and P. OHLIN.

Received 1 June 1957.

Sensitization of salivary glands by denervation has been studied particularly in cats, and to some extent in dogs also. In the present experiments sensitization to chemical agents was investigated in salivary glands of rabbits. The responses of the normal glands of this species had, however, first to be studied in some detail, since our knowledge in this field is very restricted. This is probably so because the glands are sensitive to exposure (LANGLEY 1879—80) and the ducts are tiny and rather difficult to find, at least the duct of the submaxillary gland. In most of the earlier experiments mixed saliva has been collected from the mouth, disregarding the responses of the separate glands.

Methods.

The observations were made in 41 rabbits, anaesthetized with urethane (2 g/kg) given into an ear vein. Fine glass cannulae were inserted into the salivary ducts either from the mouth or into the exposed duct (the cannula used for the submaxillary duct gave about 80, that for the parotid duct about 60 drops from one ml of distilled water). Drops falling from the tip of a cannula were recorded on the smoked drum using a signal and an electromagnetic lever. In some cases the cannula was connected via plastic tubings to a 0.1 ml pipette placed in the horizontal position. The amount of saliva could then be measured with great accuracy. Drugs were injected through a cannula in an external jugular vein. When secretory nerves were stimulated, 20 supramaximal shocks/sec. were used.

Results.

The submaxillary and the parotid glands were studied. According to FAHRENHOLZ (1937) the glandula sublingualis major is lacking in rabbits.

1. *The normal responses.* The submaxillary gland was found to secrete continuously at a slow rate. In one rabbit such an incessant flow was observed for $6\frac{1}{2}$ hours. Using the pipette system the magnitude of the flow could be estimated and was found to amount to 0.0014 ml in five minutes, on the average, varying from 0.0004 to 0.0027 ml in different animals. This secretion was not abolished by section of the chorda-lingual nerve or extirpation of the superior cervical ganglion; nor was it affected by injection of dihydroergotamine or atropine. There was no such continuous flow of saliva from the parotid gland.

Acetylcholine caused a secretion from both glands, the threshold dose being 1—2 $\mu\text{g/kg}$. More longlasting effects could be obtained with mecholyl (acetyl- β -methyl-choline) and especially with pilocarpine. The threshold dose of mecholyl was about 0.5 $\mu\text{g/kg}$ in both glands. 2 $\mu\text{g/kg}$ caused a flow of 5 or 6 drops from the parotid gland but only one or two drops from the submaxillary gland; this latter secretion was superimposed upon the continuous flow. The threshold dose of pilocarpine was in both glands 25—50 $\mu\text{g/kg}$. After 0.5—1 mg/kg the glands secreted at a maximal rate. A flow of about 0.7 ml/min. was then obtained from the parotid gland, and the submaxillary gland produced about 0.2 ml/min. A flow of similar size was obtained from the submaxillary gland by stimulation of the parasympathetic secretory fibres. This was achieved by placing the electrode on the submaxillary duct; stimulation of the chorda-lingual nerve is usually without secretory effect since the small chorda is very easily torn from the stem in the course of the dissection (RAHN 1851).

Adrenaline, even in doses of 100 $\mu\text{g/kg}$, had a very poor secretory effect on the submaxillary gland or none at all. Similarly, stimulation of the preganglionic sympathetic fibres in the neck produced a very small flow of saliva from this gland. The most striking effect of sympathetic stimulation was in fact an inhibition of secretion produced by a big dose of *e. g.* pilocarpine (fig. 1 A); this effect was probably due to a vasoconstrictory effect of the sympathetic stimulation.

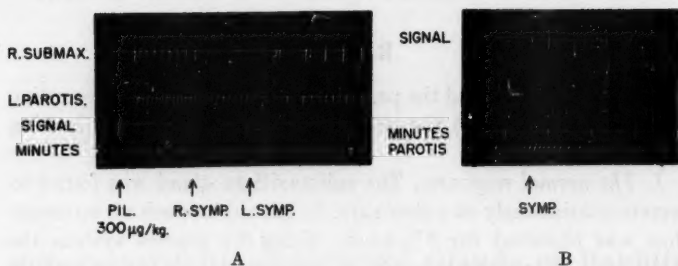


Fig. 1 A. Rabbit 2.4 kg. The inhibitory effect of sympathetic stimulation on rapid secretion after injection of pilocarpine on the submaxillary and the parotid gland. Pilocarpine 400 $\mu\text{g/kg}$ was given some minutes before the 300 $\mu\text{g/kg}$ indicated by the arrow.

Fig. 1 B. Rabbit 2.7 kg. The accelerating effect of sympathetic stimulation on slow secretion after injection of pilocarpine on the parotid gland. Pilocarpine 450 $\mu\text{g/kg}$ was given several minutes before the stimulation.

In the parotid gland adrenaline or sympathetic stimulation was found to cause a lively secretion; even big doses (up to 7 mg) of dihydroergotamine could not abolish this secretion. The threshold dose of adrenaline was about 10 to 20 $\mu\text{g/kg}$. Sympathetic stimulation evoked a secretion of 2–3 drops per minute. If the gland was already secreting because of an injection of *e. g.* pilocarpine the effect of sympathetic stimulation varied with the rate of flow. If the flow was slow, it could be accelerated by stimulation of the sympathetic fibres (fig. 1 B). A rapid flow, on the other hand, caused by a big dose of pilocarpine, was decreased or abolished by sympathetic stimulation (fig. 1 A), very likely because of vasoconstriction in the gland.

It was regularly observed that the secretory effects tended to decrease in both glands when secretory drugs were injected or secretory nerves stimulated repeatedly. In this respect the salivary glands of the rabbit differ strikingly from those of the cat and the dog.

2. *Sensitization by denervation.* Since the parotid gland was found to give the best secretory responses, this gland only was used for experiments on sensitization.

Two to three weeks after extirpation of the superior cervical ganglion, the parotid gland exhibited a supersensitivity to chemical stimuli. In one rabbit, for instance, 2 $\mu\text{g/kg}$ of mecholyl produced 12 drops from the denervated, but only 6 from the

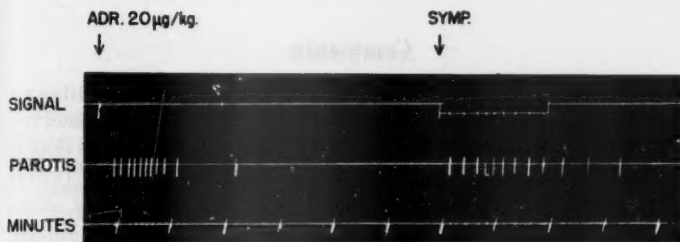


Fig. 2. Rabbit 2.3 kg. The effect of adrenaline or sympathetic stimulation on the sensitized parotid gland after treatment with Hoechst 9980 1 mg/kg daily for 3 weeks. Such an effect was never found in untreated animals.

contralateral parotid gland. With pilocarpine, likewise, a sensitization could be demonstrated; with adrenaline and noradrenaline, on the other hand, the effects were less striking.

Attempts were made to find parasympathetic secretory fibres of the parotid gland. According to RAHN (1851), secretory fibres are present in ramus III nervi trigemini and in the nervus facialis. In the present experiments the following nerves were stimulated: n. facialis, n. lingualis, chorda tympani in cavum tympani; electrodes were in addition put on the salivary duct. A small secretion was obtained in one case only, when the facial nerve was excited; such an effect could, however, not be reproduced in subsequent experiments.

It was, therefore, necessary to abstain from experiments on supersensitivity after parasympathetic denervation. Instead, experiments were carried out on animals treated for some time with a parasympathetic agent. Such a drug (Hoechst 9980)¹ has been found to cause a supersensitivity of salivary glands of cats (EMMELIN and HENRIKSSON 1953). One mg/kg of this drug was injected subcutaneously once a day over a period of three weeks. The threshold dose of adrenaline was found to be lowered from its normal value, 10–20 $\mu\text{g/kg}$, to 1–2 $\mu\text{g/kg}$, and the ordinary threshold dose caused a flow of about 10 drops of saliva. The effect of sympathetic stimulation was also remarkably big in these rabbits; thus the secretory response was more than double that of untreated animals. Fig. 2 shows some results obtained after treatment with Hoechst 9980.

¹ This drug was kindly supplied by A. B. Webass, Gothenburg.

Comments.

The submaxillary gland of the rabbit shows a continuous secretion; the observation that this is not abolished by denervation or injection of dihydroergotamine or atropine suggests that it is an example of spontaneous secretion. Also TAKAHASI (1944) observed an incessant flow of saliva from the submaxillary gland of the rabbit, using, however, awake and etherized animals. Spontaneous secretion has been found to occur in the sublingual but not in the submaxillary gland of the cat (EMMELIN 1953). It is interesting to note in this connection that the rabbit has no sublingual gland.

The parotid gland of the rabbit seems to be an organ well suited for experiments on salivary secretion. It responds well to parasympathicomimetic drugs such as mecholyl or pilocarpine. Unlike the parotid gland of the cat or the dog it also responds well to adrenaline or sympathetic stimulation. It is therefore useful for experiments in which a parasympathicolytic agent is used to produce a supersensitivity. It is supplied by sympathetic secretory fibres (this was shown by VON WITTICH 1868 and HEIDENHAIN 1878, but denied by GRUENHAGEN 1868 and JAENICKE 1878). We have been unable to find any parasympathetic secretory fibres; our observation that treatment with Hoechst 9980 causes a supersensitivity indicates, however, that the parotid gland is supplied with cholinergic fibres from some source.

Summary.

Secretion from the submaxillary and parotid glands has been studied in rabbits under urethane anaesthesia. The submaxillary gland, but not the parotid, shows a continuous secretion, which probably occurs spontaneously. The secretory responses of the glands to various drugs have been investigated. The parotid gland of the rabbit seems to be a valuable substrate for experiments on supersensitivity to chemical agents. Supersensitivity has been produced by extirpation of the superior cervical ganglion or treatment with the parasympathicolytic agent Hoechst 9980.

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Effect of Anastomosis Between the Hypoglossal and Chorda-Lingual Nerves on the Supersensitivity of the Denervated Submaxillary Gland.

By

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The cause of the supersensitivity which develops in a structure after denervation has been much discussed (see CANNON and ROSENBLUETH 1949). The efferent nerve has been supposed to exert some influence on the cells, the removal of which should result in an enhanced responsiveness to chemical stimuli. It would be interesting to know whether such a restraining influence is specific to the nerve normally innervating a structure or can be exerted by some other nerve replacing the ordinary one. Since the classical experiments of FLOURENS (1828) it is known that functional union can be established between certain nerves. DALE (1934) analysing the results of previous workers, particularly LANGLEY and ANDERSON, concluded that cholinergic fibres can be cross-sutured to each other, reinnervating end-organs or ganglia, and that, similarly, adrenergic fibres can be functionally interchanged with each other.

The present experiments were carried out on the submaxillary gland of the cat. This structure was considered a suitable preparation since it is possible to observe its level of sensitivity to chemical agents over long periods (EMMELIN and MUREN 1952); furthermore, it has been shown (in an experiment on a dog) that functional union can be established between the hypoglossal nerve

and the parasympathetic secretory fibres of the gland (CALUGAREANU and HENRI 1900, 1901).

After section of the hypoglossal and chorda-lingual nerves of one side the central stump of the former nerve was connected to the peripheral stump of the latter one. The sensitivity of the gland towards adrenaline was estimated at intervals both on the operated and the normal side. Some months after the operation the hypoglossal nerve was stimulated electrically in an acute experiment. For the sake of comparison some experiments were carried out in which the chorda-lingual nerve was cut or crushed and allowed to regenerate.

Methods.

In order to study the sensitivity of the submaxillary glands the cats were anaesthetized with evipan, given intracardially after preliminary ether. The ducts of both submaxillary glands were cannulated from the mouth using fine glass cannulae. Adrenaline was injected intracardially to induce salivary secretion and the drops of saliva falling from the cannulae were counted. In some cases, in which special attention was paid to the rate of secretion, the intervals between the drops were registered using a drop recorder. After the experiment the cat was allowed to wake up.

Cross-suturing of the nerves was made in nembutal anaesthesia. The right hypoglossal and chorda-lingual nerves were exposed and a stretch of each nerve dissected. Both nerves were cut and the central stump of the hypoglossal nerve attached to the distal one of the chorda-lingual nerve using one 6-0 silk suture (Ethicon). In some instances a branch of the hypoglossal nerve was used instead of the whole trunk. The central stump of the chorda-lingual nerve was sewn into the digastric muscle in order to prevent the secretory fibres from regenerating towards the gland.

The acute experiment was carried out in chloralose anaesthesia (about 80 mg/kg intravenously after ether). Both submaxillary ducts were exposed in the neck and cannulated. The right sublingual duct was cannulated as well. The hypoglossal nerves were cut as far centrally as possible in the neck and the peripheral stumps used for stimulation. Both sympathetics, and the left chorda-lingual nerve, were dissected for stimulation. The drops of saliva were recorded on the smoked drum using an electrical signal. The drugs were injected through a cannula inserted in a femoral vein. In some cases the flow of blood through the right submaxillary gland was measured. After injection of heparin a cannula was introduced into the right external jugular vein, draining the blood from the gland; branches of the vein from other sources had been tied. The drops of blood falling from the cannula were recorded by a phototube operating an ordinate recorder.

Results.

1. Union of the hypoglossal to the chorda-lingual nerve.

The sensitivity. The hypoglossal nerve was sewn to the chorda-lingual one in 16 cats. Ten of these survived for a period sufficiently long to allow a study of the effect of the cross-suture on the sensitivity of the submaxillary gland to adrenaline.

The effect of section of the chorda-lingual nerve is the development of a marked supersensitivity of the gland to adrenaline. After about three weeks a maximal degree of sensitivity is reached, and according to previous experience this level will be maintained indefinitely provided that regeneration of the chorda fibres does not take place; if the sensitivity is expressed in *e. g.* the number of drops of saliva produced after a moderate dose of adrenaline the response will usually decrease somewhat from its maximum, not because of a diminished sensitivity but a partial atrophy of the gland.

When, however, the peripheral end of the nerve had been joined to the central stump of the hypoglossal nerve the result was different. In all the ten cats the sensitivity started to decline. The fall could first be seen five to six weeks after the operation. The accuracy of this estimation will obviously depend on the length of the interval between the experiments in which the sensitivity was studied. The following ten pairs of figures correspond to the ten experiments, the first figures showing the last time, in days after the operation, at which the sensitivity was still found at its maximum, the second one the time of the first experiment at which a definite fall in sensitivity was observed: 23—37, 25—38, 25—40, 33—46, 24—48, 36—49, 36—50, 39—54, 32—60, 37—82.

Fig. 1 illustrates the course of the sensitivity in one of these experiments. 23 days after the cross-suture operation the sensitivity was high as judged both from the secretory response to a standard dose and the threshold. 37 days after the operation the responsiveness was much decreased and in the course of the following month there was some further fall in sensitivity. In some experiments the decline was more gradual. In one case the curve was still falling off in the sixth month after the suture; in another there was no further reduction after about 10 weeks. The observation period was in no instance longer than about half a year.

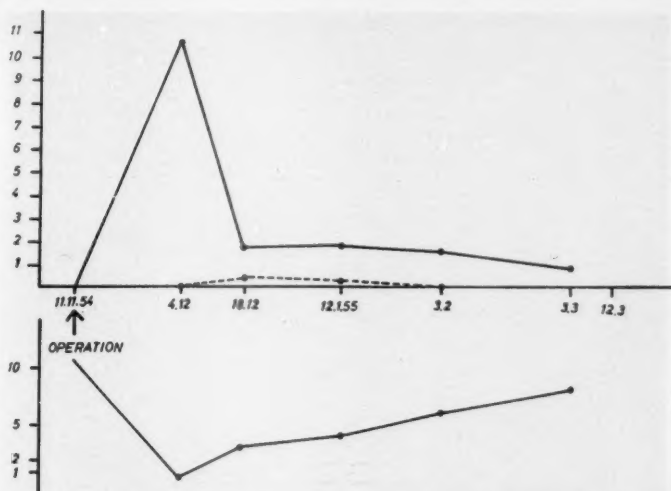


Fig. 1. Sensitivity of the submaxillary gland towards adrenaline after nerve section and union of the peripheral stump of the chorda-lingual nerve to the central stump of the hypoglossal nerve (at the arrow). The sensitivity is expressed upwards as number of drops of saliva in response to $10 \mu\text{g/kg}$ adrenaline, downwards as approximate threshold dose of adrenaline in $\mu\text{g/kg}$. The broken line shows the responses of the contralateral gland.

In none of the cases did the sensitivity completely return to its preoperative level. Table 1 demonstrates how far the restoration of the original level of sensitivity had proceeded in the different experiments.

The acute experiment. Five of the ten cats died in the course of the observation period and the remaining five were used for acute experiments in chloralose anaesthesia. In all these experiments electrical stimulation of the peripheral end of the hypoglossal nerve cut acutely as far centrally as possible to the point of suture caused a flow of saliva from the submaxillary gland. Saliva appeared quickly when stimulation started; the latency seemed to be about the same as that seen on stimulation of the left chorda-lingual nerve. As can be seen in fig. 2 some drops fell quickly, and the rate of secretion then decreased to a level which remained fairly constant on continued stimulation. In this respect the effect differs from that obtained on chorda stimulation; in the latter case the rate of secretion scarcely decreases during a stimulation period of some minutes. As an example the following

Table 1.

Cat no.	Standard dose, $\mu\text{g/kg}$	Response at maximal sensitization, drops		Last observed response, drops		Time for last observation days after op.
		Operated	Control	Operated	Control	
2	5	19	$\frac{1}{2}$	$3\frac{1}{2}$	$\frac{1}{4}$	181
3	5	10	$\frac{1}{2}$	$1\frac{1}{2}$	$\frac{1}{2}$	148
4	5	$13\frac{1}{2}$	$2\frac{1}{2}$	$1\frac{1}{4}$	$\frac{1}{4}$	163
5	5	$12\frac{1}{2}$	$\frac{2}{3}$	$7\frac{1}{2}$	$1\frac{1}{4}$	110
7	5	$12\frac{1}{2}$	$\frac{2}{3}$	$1\frac{1}{2}$	$\frac{1}{4}$	98
8	10	$10\frac{2}{3}$	0	$\frac{1}{4}$	0	112
9	5	5	0	$2\frac{1}{2}$	1	132
11	5	$15\frac{1}{2}$	$\frac{1}{2}$	$6\frac{1}{2}$	$\frac{1}{2}$	71
18	2	16	$\frac{1}{4}$	$1\frac{3}{4}$	0	109
36	10	14	$\frac{1}{4}$	4	$\frac{1}{4}$	97

observation may be quoted. Stimulation of the hypoglossal nerve was in one case found to give 10 drops in one minute, but only 22 drops in five minutes; the corresponding effects of stimulation of the left chorda-lingual nerve were 31 and 152 drops respectively. Once the secretion had decreased during the first minute, however, it was maintained at a fairly constant rate, as shown in fig. 2, contrary to the effect usually seen on stimulation of the sympathetic nerve; in that case the secretion usually ceases entirely in spite of continued stimulation or remains at a slow and irregular rate.

It was noticed that relatively strong stimuli were required to excite the nerve fibres. The secretory response tended to increase in the course of a day's experiment. In one case, for instance, only six drops were obtained during 15 minutes stimulation at the beginning of the experiment; some hours later a similar amount was produced in one minute.

The magnitude of the response also depended on the rate of stimulation. In the experiment of fig. 2, for instance, stimulation of the hypoglossal nerve at 50/sec. during five minutes caused a flow of 21 drops from the submaxillary gland; at 20/sec. only 5 drops were obtained in the same period. The relationship between frequency of stimulation and size of response can be seen in fig. 3 A, which in addition for comparison shows a similar analysis



Fig. 2. Records from above: drops of saliva from the right submaxillary gland; from the right sublingual gland; signal; time in minutes; blood flow through the right submaxillary gland, recorded with a drop recorder. The right hypoglossal nerve (sewn to the chorda-lingual nerve six months earlier) was stimulated at a frequency of 50/sec. at the first mark of the signal, at 20/sec. at the last mark.

on the chorda-lingual nerve of the control side in the same cat. There is a striking difference between the two nerves, the maximum of the chorda-lingual nerve being at about 10 stimuli/sec., that of the hypoglossal nerve at about 50/sec.

A difference between the two nerves was also observed when the duration of each stimulus was varied; an optimal rate of stimulation was used (50 and 10/sec., respectively). This can be seen in fig. 3 B. When the duration was 0.05 millise., stimulation

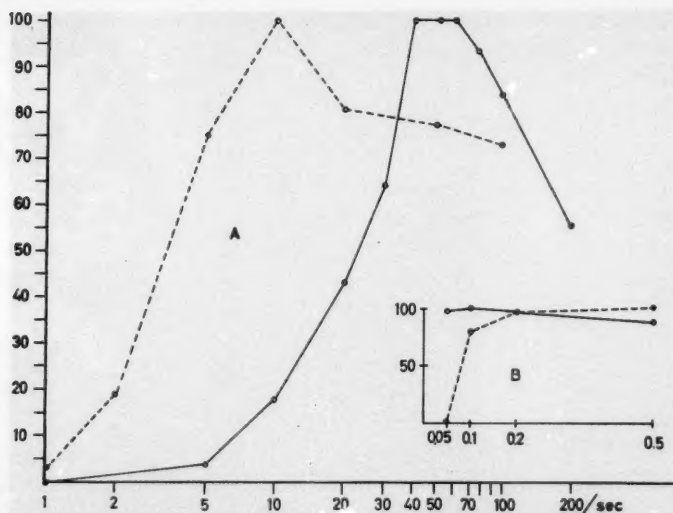


Fig. 3. A: relation between frequency of stimulation (abscissa) and response (ordinate). Solid line: stimulation of the right hypoglossal nerve (cross sutured 4 months earlier). Broken line: stimulation of the left chorda-lingual nerve. The secretory effects are given in per cent of the maximal response for each nerve. Duration of stimuli: 0.5 millisecc.

B: the duration of the stimulus was varied between 0.05 and 0.5 millisecc. Frequency of stimulation kept constant (50/sec. for hypoglossal, 10/sec. for chorda-lingual nerve).

of the chorda-lingual nerve had a very small effect only, whereas stimulation of the hypoglossal nerve caused the maximal response.

A secretory response on hypoglossal stimulation was obtained from the sublingual gland as well, superimposed upon its spontaneous secretion (EMMELIN 1953). The size of the response from this tiny gland was small, but in two of the cats it was remarkably big, compared with that of the submaxillary gland, indicating a more complete reinnervation of the sublingual gland. In one of these cases, for instance, the sublingual gland gave 4 drops and the submaxillary gland 11 drops in five minutes. A stimulation rate of 50/sec. had a bigger effect than had 20/sec. on this gland also, as can be seen in fig. 2.

A necessary control experiment seemed to be to stimulate the hypoglossal nerve of the intact side. When this was done, no secretion whatsoever was obtained. It could be hypothesized that the hypoglossal nerve normally contains a small number of

secretory fibres for the salivary glands and that the secretory effect of stimulation can only be detected when the nerve impulses act on a gland, sensitized by previous section of the chorda-lingual nerve. A special control experiment was carried out in a cat, the submaxillary gland of which had acquired a particularly high degree of sensitivity both to adrenaline and acetylcholine, after the chorda-lingual nerve had been severed. No secretion was obtained on stimulating the normal hypoglossal nerve of that side.

Stimulation of the hypoglossal nerve, cross-sutured to the chorda-lingual nerve, was found to cause vasodilatation in the corresponding submaxillary gland. This is shown in the experiment of fig. 2. During hypoglossal stimulation the flow of blood through the gland increased considerably, particularly when the higher rate of stimulation was used.

Atropine in small doses was found to abolish the secretory effect of hypoglossal stimulation. A vasodilator response was, however, retained. In these respects the results thus resembled those obtained on stimulation of the chorda tympani.

It should be realized that the secretory fibres of the chorda, cut and connected to the hypoglossal nerve, are preganglionic. In order to investigate how far the regenerating hypoglossal fibres had grown towards the glands, the effect of hexamethonium on the secretion caused by hypoglossal stimulation was studied. The drug, given intravenously, was found to abolish the effects of hypoglossal stimulation both on the submaxillary and the sublingual glands. It was observed that hexamethonium was very potent in this respect; minimal doses, far below those affecting the responses to sympathetic or chorda stimulation, were found to abolish the effects of hypoglossal stimulation, and when bigger doses were given the blocking effect was of very long duration. A dose of hexamethonium as small as 0.01 mg/kg reduced the effect of hypoglossal stimulation from 10 to 5 drops in one experiment; after about 0.03 mg/kg the response was only 2 drops, whereas neither the effect of chorda nor sympathetic stimulation was diminished. Complete recovery had taken place in about one hour. In another experiment 0.1 mg/kg was found to abolish the effect of hypoglossal stimulation for one hour and a half. The effects of stimulating the sympathetic or the contralateral chorda were not affected. Fig. 4 shows the effect of a dose of hexamethonium ordinarily used to block the ganglionic transmission.

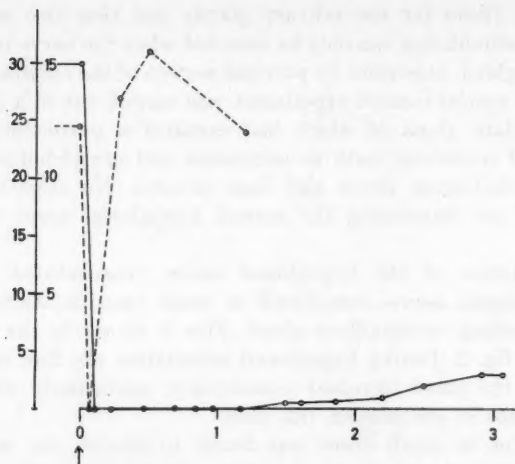


Fig. 4. Effects of hexamethonium on the secretory responses to stimulation of the right hypoglossal nerve (solid line; stimulation for one minute at 50/sec.) and of the left chorda-lingual nerve (broken line, one minute stimulation at 10/sec.). Time in hours. Ordinate: drops of saliva, to the right on hypoglossal and to the left on chorda-lingual stimulation. At zero time (arrow) intravenous injection of 5 mg/kg hexamethonium.

In one cat an attempt was made to demonstrate the release of acetylcholine by impulses sent through the hypoglossal nerve to the gland. Both submaxillary glands were perfused with eserized cat plasma. Unfortunately the nerve was stimulated using an unfavourable frequency, 20/sec., and only a trace of saliva was obtained. The content of acetylcholine in the venous effluent rose from 0.002 μg in the resting state to 0.006 μg during two minutes of such stimulation. In the normal gland, stimulated through the chorda, it increased to 0.190 μg .

Sensitivity and effect of hypoglossal stimulation. Table 2 summarizes the secretory effects of stimulation of the right hypoglossal, the left chorda-lingual, and the left sympathetic nerve. The figures are number of drops obtained during one minute. The two normal secretory nerves were stimulated at a frequency of 20/sec.; to the hypoglossal nerve 50 stimuli were applied per second, and the responses given are the biggest ones obtained, *i. e.* those found late in the experiment. It can be seen from the table that the secretory effects of hypoglossal stimulation were never

Table 2.

Cat no.	Right hypogl.	Left chorda	Left symp.	Weight of the glands	
				right	left
2	12	27	7 $\frac{1}{2}$	—	—
4	7	—	7 $\frac{1}{2}$	0,615	0,875
8	14	26 $\frac{2}{3}$	1 $\frac{1}{3}$	—	—
9	4	24 $\frac{2}{3}$	6	0,511	0,942
18	9 $\frac{1}{4}$	31	7	0,876	1,418

as big as those produced by the corresponding normal parasympathetic fibres; at most they were about half that size. It is reasonable to connect this with the finding that the supersensitivity created by section of the chorda-lingual nerve was in no case fully abolished. The smallest secretion on hypoglossal stimulation was found in cat no. 9; in table 1 it can be seen that the decrease in sensitivity was fairly small in this particular cat.

A comparison with the responses to stimulation of the normal sympathetic nerve may also be of some interest. In three of the cats (4, 9, 18) the sympathetic nerve could produce about the same number of drops in one minute as could the artificial secretory nerve; nevertheless, the sympathetic fibres were apparently unable to counteract the development of the supersensitivity when the chorda-lingual nerve had been transected, whereas the outgrowing hypoglossal fibres had such an ability.

The weights of the glands. In three of the cats the submaxillary glands were weighed at the end of the experiment. In all the three cats the right gland was found to be smaller than the left one. This difference should be kept in mind when comparing the secretory responses of the two glands (table 2).

2. Regeneration of the chorda tympani.

The chorda-lingual nerve of the right side was crushed with an artery forceps in two cats, and severed in seven cats; in three of these latter cases the cut ends were united with a suture. The results of these experiments are summarized in table 3. Some of the experiments were originally intended for other investigations; that is the reason why some important data are lacking.

Table 3.

Cat no.	Chorda-lingual	Standard dose of adr.	Response at max. sens.		Last response		Response to chorda-lingual nerve	
			operated	control	operated	control	operated	control
30	crushed	5	9 $\frac{1}{2}$	$\frac{1}{4}$	1	$\frac{1}{2}$	11	15
35	"	5	18 $\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{3}$	$\frac{1}{4}$	—	—
48	cut + suture	10	11	0	4 $\frac{1}{2}$	0	2	25
51	"	5	18	$\frac{1}{4}$	5 $\frac{1}{4}$	0	12	18
52	"	5	15 $\frac{1}{2}$	1	9	3 $\frac{1}{4}$	9	19
18	cut	10	—	—	4 $\frac{3}{4}$	2 $\frac{1}{2}$	17	28
36	"	20	24	4 $\frac{1}{2}$	10 $\frac{1}{2}$	$\frac{1}{2}$	—	—
16	"	5	23	3 $\frac{1}{4}$	$\frac{1}{4}$	—	5 $\frac{3}{4}$	—
67	"	5	—	—	4	4 $\frac{1}{4}$	11	10
60	"	5	19	1	3 $\frac{1}{4}$	$\frac{1}{4}$	35	38
45	"	5	20 $\frac{1}{2}$	$\frac{1}{2}$	11	0	21	54

When the chorda-lingual nerve was crushed the ensuing supersensitivity was of short duration. A decline in sensitivity was observed after between 19 and 28 days in cat no. 30, and between 19 and 32 days in no. 35. The preoperative level was almost reached within two months in both cases. The effect of chorda stimulation, tested in cat no. 30, was nearly as big on the operated as on the contralateral side.

When the nerve was cut the return of the sensitivity curve towards normal was generally less rapid and less complete. Corresponding to this, the effect of chorda stimulation was usually smaller on the operated than on the normal side. The suture, bringing together the cut ends, did not seem to favour the reinnervation process strikingly.

In cat no. 67, in which the effects of stimulation of the chorda-lingual nerves were about the same on both sides (213 days after section of the nerve), the submaxillary gland of the operated side was perfused with eserized plasma and the acetylcholine released during chorda stimulation assayed on cat's blood pressure. The output of acetylcholine during two minutes was found to be 0.150 μ g; under similar experimental conditions we have found a release

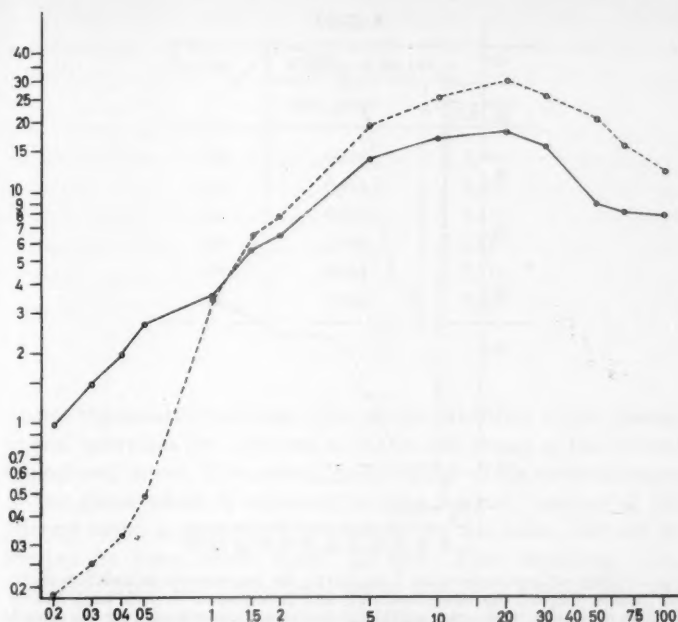


Fig. 5. Stimulation of the normal, left chorda-lingual nerve (broken line) and the partly regenerated, right chorda-lingual nerve (solid line) at different frequencies (abscissa). At low frequencies the responses were bigger on the right side, in spite of the reinnervation being incomplete, indicating sensitization. At higher frequencies the effects on the right side were smaller, partly because of atrophy of the gland but particularly because of incomplete reinnervation.

from a normally innervated gland of $0.144 \mu\text{g}$ on the average (EMMELIN and MUREN 1950).

The optimal frequency of stimulation was found to be the same for the regenerated and the normal secretory fibres. This can be seen in fig. 5 which was plotted from data obtained in the acute experiment on cat no. 18. In this cat a slight supersensitivity to adrenaline and acetylcholine was found to remain. It is interesting to observe in the figure that a supersensitivity to impulses sent through the chorda could be demonstrated as well, when low frequencies of stimulation were used.

The effect of stimulating the regenerating chorda-lingual nerve could be completely abolished by administration of hexamethonium. The drug was more effective on the operated than on the normal side, but the difference was not at all as striking as in the

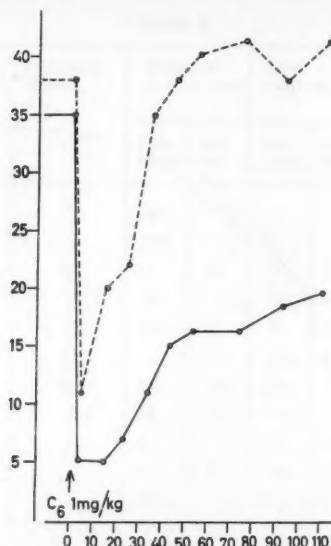


Fig. 6. Effect of hexamethonium, 1 mg/kg, on the responses to stimulation of the left (broken line) and the regenerated right chorda-lingual nerve (solid line). Abscissa: time in minutes. Ordinates: number of drops during stimulation for one minute at a frequency of 20/sec.

experiments with hypoglossal stimulation, described above. Even when extensive reinnervation had obviously occurred, hexamethonium had a more pronounced blocking effect than on the control side. In cat no. 60, for instance, stimulation of the right chorda-lingual nerve gave almost as much saliva as stimulation of the left one; nevertheless, hexamethonium had a more marked effect on the right than on the left side, as can be seen in fig. 6.

The weights of the submaxillary glands are shown in table 4. It can be seen that the right gland was almost as big as the left one in two cats, no. 35 and no. 60. Table 3 suggests that the reinnervation in these two cats had been extensive.

Discussion.

The experiments of the present paper show that the supersensitivity following section of the chorda tympani (which will remain for an indefinite period of time provided that the chorda fibres

Table 4.

Cat no.	Weights of glands, g	
	right gland	left gland
35	0.916	1.094
48	0.679	1.034
51	0.622	0.912
36	1.420	1.945
60	0.949	1.110
45	0.864	1.609

do not regenerate) decreases if the peripheral stump of the chorda-lingual nerve has been sutured to the central stump of the severed hypoglossal nerve. The restraining influence on the responsiveness of the gland which is supposed to be a normal function of the efferent nerve, is apparently not specific to this nerve, but can be exerted by some other nerve as well. That functional reinnervation from the hypoglossal nerve had been established was shown in the acute stimulation experiments. Stimulation of the hypoglossal nerve was found to cause the effects typical of chorda stimulation: secretion from the submaxillary and sublingual glands, which could be abolished by atropine, and vasodilatation, with no constrictor component, which was not abolished by atropine. There was not a complete return of the sensitivity to its low preoperative level. Correspondingly, the hypoglossal nerve was found not to be as efficient a secretory nerve as the lost chorda. This was probably due to incomplete reinnervation, for when the chorda-lingual nerve was cut and left to regenerate, the secretory effect of stimulation was usually below normal; and there was some supersensitivity left. The reinnervation in both types of experiments was probably not as extensive, in an anatomical sense, as it was functionally; it should be kept in mind that the regenerated fibres probably acted on supersensitive cells. This assumption is supported by the finding that the regenerated chorda fibres had a bigger secretory effect than the normal ones when stimulated at a low frequency.

The supersensitive cells referred to may be the gland cells. They may, however, in addition be the postganglionic parasympathetic

ganglion cells. These cells are known to become more responsive after section of the chorda (EMMELIN 1953), and the experiments with hexamethonium showed that the outgrowing hypoglossal (or chorda) fibres reached as far as to the ganglion cells.

When the mode of stimulation was varied as to frequency and duration the hypoglossal fibres were found to differ strikingly from the normal or regenerating chorda fibres. It seems reasonable to assume that the fibres of the hypoglossal nerve, the stimulation of which caused secretion and vasodilatation, were the somatomotor fibres intended for the muscles of the tongue. According to DALE, FELDBERG and VOGT (1936) these fibres are cholinergic. Accepting the view that only a cholinergic nerve functionally can replace another cholinergic nerve in a cross-suturing experiment, the present results support previous findings (EMMELIN and MUREN 1950) in favour of the opinion that the preganglionic chorda tympani fibres are cholinergic.

Relatively strong stimuli were found to be necessary to excite the regenerated nerves. It is interesting to note that already HOWELL and HUBER (1892) observed that "conductivity in a regenerating nerve returns before excitability".

The incompleteness of the reinnervation is shown not only in the remaining supersensitivity and the smallness of the secretory response to stimulation. The atrophy of the gland, caused by section of the chorda, still persisted except in those cases in which the reinnervation (from the chorda) had been particularly good. The bigger effect of hexamethonium may likewise be due to the reinnervation being only partial. Acetylcholine may be released from the hypoglossal or regenerated preganglionic chorda fibres in small amounts only, with little margin of safety; the falling off of the secretory response at the beginning of a period of hypoglossal stimulation may be explained in that way. It is reasonable to suppose that the synapse should be more susceptible to the blocking action of hexamethonium under such circumstances. It is interesting to find that even if the reinnervation from the chorda seemed complete, as judged from the secretory response, the level of supersensitivity, and the weight of the gland, addition of hexamethonium could reveal that normal conditions were not quite restored; the safety factor as regards acetylcholine release may still have been too small. It was also interesting to see that the heterogeneous synapse was particularly easily blocked by hexamethonium.

The results of the present investigation suggest that repeated observations on the level of sensitivity of an organ towards suitable chemical agents may provide a useful method for studies on regeneration of nerve fibres. Regeneration is announced by decline in sensitivity, and the progress of the regeneration process can be watched on the sensitivity curve. The sensitivity level finally reached gives a measure of the degree of (functional) reinnervation attained; and further information as to the completeness of regeneration may be obtained by the use of suitable drugs.

Summary.

The central stump of the hypoglossal nerve was connected to the peripheral stump of the chorda-lingual nerve in cats, and the effect of this operation on the sensitivity of the submaxillary gland towards adrenaline was studied.

The supersensitivity caused by the denervation of the gland was in these cases not found to remain on its high level but started to decrease after five to six weeks. When the hypoglossal nerve was stimulated electrically later on in an acute experiment the effects typical of stimulation of the chorda tympani ensued: secretion from the submaxillary and sublingual glands, which was abolished by atropine; and a vasodilatation in the submaxillary gland, which persisted after injection of atropine. Hexamethonium was remarkably efficient in abolishing these effects of hypoglossal stimulation.

It is concluded that the fall in sensitivity was due to reinnervation of the gland from the hypoglossal nerve, indicating that the restraining influence on the sensitivity, which seems to be a function of the normal efferent nerve, is not specific to this nerve but can be exerted by other nerve fibres as well. In this particular case the effect of the parasympathetic secretory fibres was taken over by somatomotor fibres meant for the tongue.

The investigation was supported by a grant to one of us (N. E.) from the Swedish Medical Research Council.

Technical assistance was given by Miss URSULA DELFS.

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Effect of Anastomosis Between the Hypoglossal and Sympathetic Nerves on the Supersensitivity of the Denervated Submaxillary Gland.

By

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Stimulation of the sympathetic trunk in the neck is in most cats followed by an abundant flow of saliva from the submaxillary gland. When the parasympathetic secretory pathway is broken by section of the chorda tympani, a pronounced supersensitivity to chemical agents appears in the gland. This is the case even if the sympathetic innervation is left intact. The fact that the sympathetic fibres are unable to prevent the development of the supersensitivity is easily explained if one accepts the common opinion that the two sets of nerves innervate different gland cells. According to BABKIN (1944), the mucous cells are supplied with parasympathetic, the serous cells with sympathetic nerve fibres. LANGENSKIÖLD (1941) concluded, however, from experiments on the electrograms of the gland, that "all secretory cells in the submaxillary gland that are innervated by the sympathetic are also innervated by the chorda". Some arguments supporting this latter view may be quoted. Although the sympathetic fibres usually have a pronounced secretory effect when stimulated alone, they are unable to increase the rate of secretion when the chorda fibres are causing their maximal secretory response (EMMELIN 1955); with a microelectrode inserted into a single secretory cell, electrical responses can be obtained both on stimulation of the chorda and of the sympathetic fibres (LUNDBERG 1955); and the oxygen consumption of chopped submaxillary glands cannot be further raised by adrenaline when pilocarpine is already causing its

maximal increase (STRÖMBLAD 1957). With this evidence of a double innervation of the gland cells some other explanation of the fact that the sympathetic fibres cannot prevent the development of the supersensitivity after section of the chorda seems required. A reasonable hypothesis might be that the level of sensitivity is determined by the intensity of the impulse traffic in the nerves supplying the cells and that the flow of impulses in the secretory sympathetic fibres is scanty; it may be pointed out that the chorda tympani is the main secretory nerve and that no digestive reflexes seem to be mediated via the sympathetic pathway.

In a previous investigation it was found that if the central end of the hypoglossal nerve is connected to the distal end of the chorda-lingual nerve, the supersensitivity which is the ordinary consequence of section of the chorda does not remain at its high level but diminishes in the course of some months (EMMELIN, MUREN and STRÖMBLAD 1957). The hypoglossal nerve is thus able to counteract the supersensitivity caused by the cutting of the chorda. By connecting the hypoglossal nerve to the peripheral stump of the sympathetic instead, it would therefore be possible to test the hypothesis presented above. It is known that somatomotor fibres can form functional connection with the sympathetic in the neck; LANGLEY and ANDERSON (1904), for instance, joined the fifth cervical nerve, DUEL and BALLANCE (1932) and DE CASTRO (1934) the hypoglossal, and WOLFF, HARE and CATTELL (1938) the phrenic nerve to the cervical sympathetic trunk and observed the ocular effects of regeneration of the somatomotor fibres.

Methods.

The methods were the same as those used in the preceding paper. The sensitivity of the submaxillary glands towards adrenaline was estimated at intervals in cats under evipan anaesthesia, cannulae being inserted into the salivary ducts from the mouth and drugs given intracardially. Supersensitivity was produced by section of the right chorda-lingual nerve; the central stump of the nerve was sutured to the digastric muscle to prevent reinnervation of the gland from this source. The right hypoglossal nerve and the right sympathetic were cut and the proximal end of the former nerve connected to the distal stump of the latter using one 6-0 silk suture (Ethicon). The central stump of the sympathetic trunk was directed towards the surface by means of a suture through the subcutaneous tissue. This operation was made in nembutal anaesthesia. In the final, acute experiment chloralose was used.

In three experiments the vagus was used instead of the hypoglossus, and in six experiments the phrenic nerve was used. Unfortunately eight of these cats died within a week after the operation. One cat with the phrenic nerve connected to the sympathetic survived until the acute experiment.

Results.

The hypoglossal nerve was sutured to the sympathetic in 24 cats; 14 of these survived for such a period of time as to allow repeated observations on the sensitivity, and 8 out of the 14 cats were finally used for acute experiments.

The sensitivity. In three of the 14 experiments no decrease in sensitivity occurred; in the others there was a more or less pronounced fall. Sometimes the fall of the sensitivity curve was steep. Such an experiment is shown in fig. 1. In other cases it was more gradual and it was thus difficult to state when a definite fall had first taken place. In those cases in which this was possible, the following figures were obtained; the first figure of each pair given below shows the last time, in days after the operation, at which the sensitivity was still maximal, the second one the first time a fall was observed: 22—52, 40—55, 28—58, 45—71, 54—77, 46—87, 46—87, 62—93, 63—94.

As a result of the section of the sympathetic trunk the pupil of that side was of course narrow and the nictitating membrane relaxed, and both structures were supersensitive to adrenaline. In the course of the observation period some return towards normal was usually observed, both in position and sensitivity of the structures. At about the same time it could be seen that dilatation of the pupil and contraction of the nictitating membrane occurred when the cat licked its paw or swallowed, for instance when a rubber tube was pushed through the mouth into the pharynx. This effect was first seen at about the time when a decline in sensitivity of the submaxillary gland was observed (fig. 1). In the beginning the response was feeble; the effect on the pupil could only be detected when the eye was protected from strong light. In the course of time it grew more marked and in some cats maximal dilatation of the pupil, even in bright light, and complete retraction of the membrane occurred on licking or swallowing.

In some cats the pupillary response was pronounced and that of the membrane weak, in others the opposite was true. Fig. 2

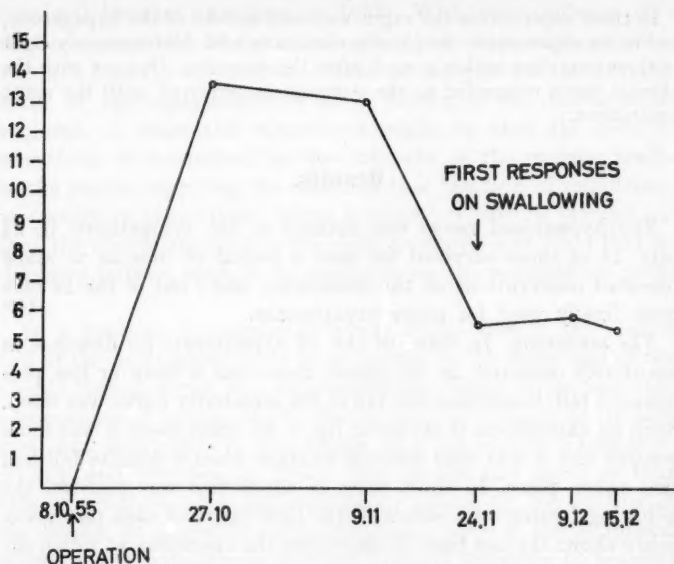


Fig. 1. Cat no. 33. Secretory effects (in drops) of $2 \mu\text{g/kg}$ adrenaline. The right hypoglossal nerve was sewn to the sympathetic trunk in the neck and the chorda-lingual nerve cut (at the arrow). On the contralateral side this dose of adrenaline evoked no secretion.

shows the effects of swallowing on the pupil and the membrane in one of the cats.

Table 1 summarizes the observations on the sensitivity in the different cats.

In one cat under evipan anaesthesia a fine polythene tube was inserted into the submaxillary duct from the mouth. When the anaesthesia began to wear off swallowing was found to be followed by secretion of a small amount of saliva as well as dilatation of the pupil and retraction of the nictitating membrane.

The acute experiment. Stimulation of the peripheral stump of the right hypoglossal nerve, cut acutely as far centrally as possible to the point of cross-suture gave a pronounced secretion from the submaxillary gland in four cats. In the remaining four animals very little or no saliva was obtained. Table 2 shows the secretory responses (obtained in one minute) at their heights; at the outset of the experiment the effects were often somewhat smaller and

Table 1.

Cat no.	Standard dose of adrenaline, $\mu\text{g/kg}$	Response at maximal sensitization		Last observed response		time for last response days
		operated	control	operated	control	
32	5	21	$1\frac{1}{4}$	8	$1\frac{1}{4}$	230
24	2	16	0	$\frac{1}{3}$	0	83
33	2	$13\frac{2}{3}$	0	$5\frac{1}{3}$	0	76
43	2	8	0	$\frac{2}{3}$	0	177
22	5	12	2	$10\frac{1}{3}$	$\frac{1}{3}$	113
57	5	$19\frac{1}{3}$	0	17	$\frac{1}{4}$	136
45	2	10	0	$1\frac{1}{4}$	0	170
60	5	19	1	$3\frac{1}{4}$	$\frac{1}{4}$	134
15	5	$30\frac{2}{3}$	$2\frac{1}{3}$	14	0	110
16	5	$40\frac{1}{3}$	$3\frac{2}{3}$	10	$\frac{2}{3}$	164
17	5	$16\frac{1}{3}$	0	$15\frac{1}{3}$	0	175
46	5	$16\frac{1}{3}$	5	$8\frac{2}{3}$	2	94
47	5	19	$\frac{1}{4}$	$3\frac{1}{3}$	0	184
59	2	$13\frac{1}{3}$	0	$4\frac{2}{3}$	0	112

increased in the course of the day. The sizes of the responses varied with the frequency of stimulation, as shown in table 3.

The effects of hypoglossal stimulation resembled those normally produced by stimulation of the sympathetic trunk in the following respects:

1. The rate of secretion diminished rapidly and ceased, often completely, in spite of continued stimulation. In this respect the picture differed strikingly from that obtained by stimulation of a hypoglossal nerve regenerated into the chorda tympani.

2. The secretory effect was not abolished by small doses of atropine.

3. It was abolished by a small dose of dihydroergotamine.

4. A vasoconstriction was obtained. The diminished blood flow through the submaxillary gland during hypoglossal stimulation is shown in the experiment of fig. 3.

The effects of stimulation of the hypoglossal nerve were antagonized by hexamethonium. The blocking action on the heterogeneous synapse was more pronounced and more persistent than on that of the normal chorda or sympathetic nerve, as shown in fig. 4.

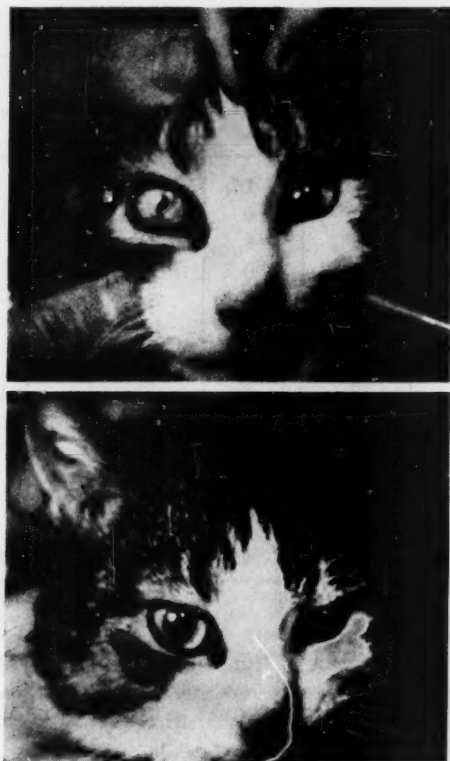


Fig. 2. Two pictures from a film of cat no. 32, 95 days after the right hypoglossal nerve had been connected to the sympathetic trunk. Upper picture: the right membrane still relaxed, partly covering the pupil and the right pupil still smaller than the left one. Lower picture: a rubber tube, introduced into the mouth, caused swallowing. This was followed by retraction of the membrane and dilation of the pupil.

On hypoglossal stimulation salivary secretion was obtained from the sublingual gland also. In one cat a small response was produced from the parotid gland as well.

Further, the pupil dilated and the nictitating membrane contracted when the hypoglossus was stimulated. Corresponding to the observations previously made on these structures during licking or swallowing movements, it was found that the response to electrical stimulation was sometimes more pronounced from

Table 2.

Cat no.	Hypoglossal stimulation 50/sec.	Left sympathetic 20/sec.	Reaction of pupil and for membrane
32	$13\frac{1}{2}$	10	+
24	$6\frac{3}{4}$	6	+
33	$6\frac{1}{2}$	$14\frac{1}{2}$	+
43	6	6	+
22	0	9	—
57	$\frac{1}{4}$	4	+
45	$\frac{1}{4}$	$5\frac{3}{4}$	+
60	0	—	+

the pupil, sometimes from the membrane. Even if the response was as big as that obtained on the opposite side by excitation of the left sympathetic it was not at all as well maintained during a stimulation period of one minute.

The effects of hypoglossal stimulation on the pupil and the membrane were often small at the beginning of the acute experiment and increased in the course of the day. This was the case even if the reflexly evoked responses in the non-anaesthetized cat had been pronounced.

In two cats the transected sympathetic was found to have regenerated. In one of these cats (no. 33) stimulation of the sympathetic far centrally to the point where it had once been cut produced a flow of 11 drops of saliva from the submaxillary gland in one minute; the left sympathetic gave $14\frac{1}{2}$ drops. The frequency of stimulation was in these instances 20/sec.; when it was diminished to 5, 3 or 2 the right nerve was found to produce a bigger response than the left one because of acting on supersensitive cells. When the right sympathetic was excited in this cat the pupil dilated maximally but the nictitating membrane was not affected. Swallowing had elicited a big contraction of the membrane but no dilatation of the pupil, and electrical stimulation of the hypoglossal nerve had the same effect. Similar observations were made in cat no. 32, although in this case the right hypoglossus had reinnervated the pupillary dilator also.

Sensitivity and effect of hypoglossal stimulation. Table 2 shows that the secretory effects of hypoglossal stimulation were pro-



Fig. 3. Cat no. 24. Blood flow through the right submaxillary gland, recorded using a phototube and an ordinate recorder. Time: minutes. At the signal the right hypoglossal nerve was stimulated; frequency 50/sec.

nounced in four cats and very small or absent in four. A comparison with table 1 shows that in the former cats the sensitivity of the gland had decreased considerably. It had, however, not reached its preoperative level; in this connection it may be pointed out that the hypoglossal responses were markedly smaller than those normally obtained on stimulation of the chorda tympani. They were, on the other hand, of the same order of magnitude as those evoked by sympathetic stimulation.

The remaining four cats have to be discussed separately. In cat no. 22 the hypoglossal nerve did not give any secretion. No decrease in sensitivity had occurred (the slightly smaller response to the standard dose of adrenaline being explained by atrophy of the gland). In cat no. 57 not more than $\frac{1}{4}$ of a drop could be produced even if the hypoglossal nerve was stimulated for long periods, and this response did not increase but decreased in the course of the experiment. No decline in sensitivity had taken place. In cat no. 45 no secretion occurred at the beginning of the experiment, but after 5 to 6 hours there was a response of $\frac{1}{4}$ drop. In spite of this small effect the sensitivity had declined very much. The explanation of this was found to be that the chorda tympani had regenerated although at the operation the central stump had been removed from its original position. Regenerated fibres could be seen, the stimulation of which gave 21 drops in one minute,

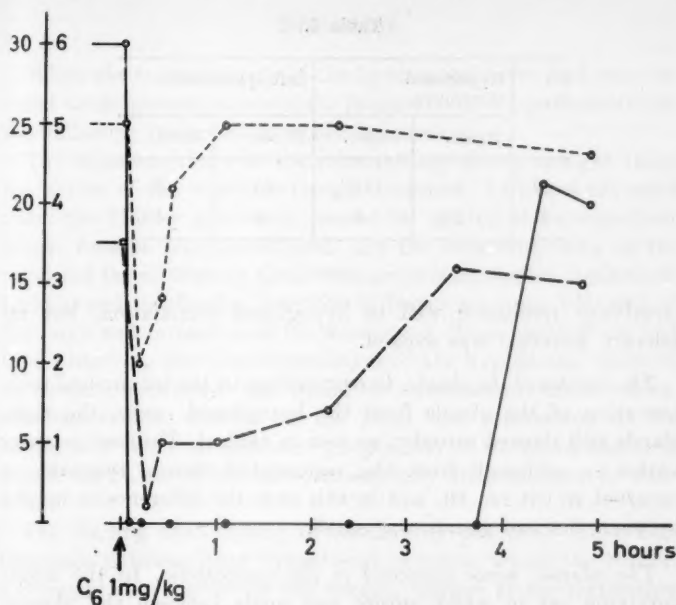


Fig. 4. Cat no. 43. Blocking effect of hexamethonium, 1 mg/kg. Ordinates: drops of saliva during one minute; the bigger figures correspond to stimulation of the left chorda-lingual nerve (20/sec., dotted line), the smaller figures to stimulation of the right hypoglossus (50/sec., solid line) and of the left sympathetic (20/sec., broken line).

whereas the left chorda produced 54 drops. In cat no. 60, finally, similar observations were made. The sensitivity had decreased markedly, but stimulation of the hypoglossal nerve gave no secretion. Regenerated chorda fibres were found, yielding 35 drops on one minute's stimulation; the left chorda gave 38 drops. Experiments no. 45 and 60 have been included in a previous paper (EMMELIN, MUREN and STRÖMBLAD 1957) as instances of chorda regeneration.

In those four cats, in which the submaxillary gland had been well innervated by hypoglossal fibres, the same was true for the pupillary dilator and for the nictitating membrane. Even in those two cats in which only a small secretory effect was obtained, hypoglossal stimulation gave the ocular effects. In cat no. 22 none of the structures had been supplied by regenerated hypoglossal fibres. In cat no. 60, however, both the pupil and the

Table 3.

Cat no.	Hypoglossal stimulation		Left sympathetic	
	20/sec.	50/sec.	20/sec.	50/sec.
32	4	13 $\frac{1}{3}$	10	3
24	2 $\frac{2}{3}$	6	6	4 $\frac{1}{3}$

membrane responded well to hypoglossal stimulation, but no salivary secretion was evoked.

The weights of the glands. Corresponding to the incomplete reinnervation of the glands from the hypoglossal nerve, the right glands still showed atrophy, as seen in table 4. The best reinnervation — although from the regenerated chorda tympani — occurred in cat no. 60, and in this case the difference in weight between the two glands was small.

The phrenic nerve connected to the sympathetic. In the single surviving cat in which suture was made between the phrenic nerve and the sympathetic the sensitivity of the gland towards adrenaline did not diminish during an observation period of 178 days. No respiratory hippus could be seen. In the acute experiment stimulation of the phrenic nerve caused no secretory or ocular responses. The submaxillary gland of the operated side showed a marked atrophy (cat no. 54, table 4).

Table 4.

Cat no.	Weights of the glands, g	
	right gland	left gland
32	0.716	1.244
24	0.590	1.059
33	0.831	1.489
43	0.549	0.992
45	0.864	1.609
60	0.949	1.110
54	0.610	1.305

Discussion.

When the central stump of the hypoglossal nerve had been sutured to the peripheral one of the preganglionic sympathetic trunk, the following observations could be made.

The supersensitivity of the submaxillary gland, brought about by section of the chorda tympani, decreased. At about the same time, the Horner syndrome, caused by cutting the sympathetic trunk, became less pronounced, and the supersensitivity of the pupil and the nictitating membrane towards adrenaline diminished. Licking and swallowing movements began to cause widening of the pupil and retraction of the membrane. When such effects had been observed, electrical stimulation of the hypoglossal nerve in an acute experiment was found to cause secretion of saliva, vasoconstriction, dilation of the pupil and contraction of the membrane. When no decrease in sensitivity of the gland had been found, excitation of the nerve caused very little secretion or none at all.

The finding that licking or swallowing produced the ocular responses indicates that hypoglossal impulses, which the central nervous system meant for the tongue, arrived at the nictitating membrane and the dilator of the pupil. The improvement of the Horner syndrome suggests that these structures became supplied by impulses which ordinarily should have given the tongue muscles a certain degree of tone. Similar observations and conclusions have been made by LANGLEY (1898), DUEL and BALANCE (1932) and DE CASTRO (1934, 1951). It seems likely that hypoglossal impulses could reach the submaxillary (and sublingual and parotid) gland as well, and in one cat it was in fact observed that swallowing was followed by salivary secretion. Sometimes the secretory response to hypoglossal stimulation was small at the start of the acute experiment and increased later. Since the same applies to the ocular responses even if they had been pronounced earlier, when elicited reflexly, it is reasonable to assume that impulses from the central nervous system could easily be transmitted along the regenerated hypoglossal nerve and that the small size of the responses in the beginning was a phenomenon connected with the artificial stimulation of the regenerated fibres. Fairly strong stimuli had to be applied to the regenerated nerve in order to evoke responses, in accordance with common experience.

A reasonable conclusion of the present experiments seems to

be that the supersensitivity of the submaxillary gland decreases because the gland receives nerve impulses via the hypoglossal nerve. The experiments with hexamethonium suggest that these impulses are transmitted through the postganglionic sympathetic neurone to the gland. The findings that hypoglossal stimulation causes secretory and vascular effects typical of sympathetic stimulation support this view. The normal sympathetic innervation is unable to counteract the sensitization caused by section of the chorda; the hypoglossal nerve, acting on the postganglionic sympathetic neurone, is able to do so, in spite of the fact that artificial stimulation has no greater secretory effect than stimulation of the normal sympathetic. The probable explanation seems to be that normally the traffic of impulses through the sympathetic fibres to the secretory cells is very small. Since it is possible to diminish the supersensitivity, which develops after section of the chorda, by directing a stream of impulses to the gland through the sympathetic pathway this must provide further evidence in support of the view that the sympathetic innervates gland cells also supplied with a parasympathetic innervation. The fact that the sensitivity does not return to its preoperative level might possibly suggest that normally the overlap in innervation is not complete; a more likely explanation, however, is that the reinnervation is incomplete. It may be pointed out that some degree of supersensitivity will persist even in experiments in which the hypoglossal has regenerated into the chorda tympani. Another fact, suggesting an incomplete reinnervation of the superior cervical ganglion from the hypoglossal nerve, is that the ocular responses were less well maintained than on ordinary sympathetic excitation. The sensitivity of the heterogeneous synapse towards hexamethonium may point in the same direction. The blocking effect of this drug was much bigger and more longlasting on the responses to hypoglossal stimulation than on those to stimulation of the normal chorda or sympathetic. It was, however, not as striking as that seen in the experiments with hypoglossal-chorda suture. In this connection it is of interest to find in a recent paper by MURRAY and THOMPSON (1957) that hexamethonium has a particularly big blocking effect on the superior cervical ganglion partly denervated by section of some of the rami communicantes but reinnervated by collateral sprouting from remaining preganglionic fibres.

Summary.

Cross nerve anastomosis was performed between the proximal end of the hypoglossal nerve and the distal end of the cervical sympathetic trunk in cats. At the same time the chorda-lingual nerve was cut. In some of the cats the supersensitivity of the submaxillary gland towards adrenaline, which follows this latter operation, was not maintained. The sensitivity started to decrease and at about the same time signs of reinnervation of the pupillary dilator and nictitating membrane from the hypoglossal nerve appeared. In acute experiments stimulation of the hypoglossus was then found to cause effects, characteristic of stimulation of the sympathetic trunk: secretion from the submaxillary gland (and sometimes from the sublingual and parotid glands as well); these responses were abolished by dihydroergotamine but not by atropine; vasoconstriction occurred in the gland; the pupil dilated and the nictitating membrane was withdrawn.

It is inferred that the hypoglossal nerve had reinnervated the superior cervical ganglion, and that impulses from the nuclei of the XIIth cranial nerve had reached the salivary gland cells via the hypoglossal and postganglionic sympathetic fibres; and that such impulses had lowered the high level of sensitivity created by section of the chorda. The observations seem to lend further support to the opinion that the gland cells innervated by sympathetic fibres have a parasymphathetic nerve supply as well.

The investigation was supported by a grant to one of us (N. E.) from the Swedish Medical Research Council.

Technical assistance was given by Miss URSULA DELFS.

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Chromatographic Separation of Five Vitamin A₁ Isomers from the Eyes of Deep-Water Prawns (*Pandalus Borealis*).

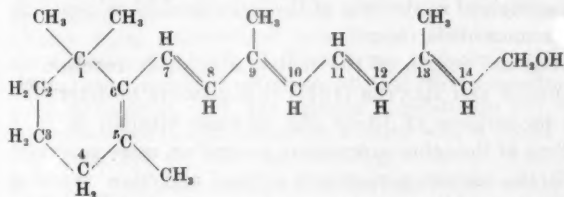
By

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During recent years a number of *cis*-isomers of vitamin A₁ have been synthesized. Our knowledge of all-*trans* vitamin A₁ and the 13-*cis* (*neo a*) vitamin A₁¹ which was isolated in 1947 by ROBESON and BAXTER has thus been supplemented with physical and chemical data on 9-*cis* and 9,13-di-*cis* vitamin A₁ (ROBESON, CAWLEY, WEISLER, STERN, EDDINGER and CHECHAK 1955), 11-*cis* (*neo b*) (DIETERLE and ROBESON 1954, OROSHNIK 1956) and 11,13-di-*cis* vitamin A₁ (WALD, BROWN, HUBBARD and OROSHNIK 1955, OROSHNIK 1956). In the case of some of these vitamin A₁ isomers determinations have also been made of the vitamin A activity of the acetates (13-*cis* vitamin A₁: HARRIS, AMES and BRINKMAN 1951, 11-*cis*, 9-*cis*, 9,13-di-*cis* vitamin A₁: AMES, SWANSON and HARRIS 1955). Unlike the four first mentioned

¹ The numbering system used in this article is that devised by Karrer:



For the sake of brevity the double bonds are in the following denoted by the number of the carbon atom which has the lowest number:

4—573465. *Acta phys. Scandinav.* Vol. 41.

isomers the two isomers, 11-*cis* and 11,13-di-*cis* vitamin A₁, are examples of vitamin A₁ isomers that possess an unstable (stereochemically ineffective) *cis* configuration (PAULING 1939, ZECHMEISTER 1944), a configuration which was previously assumed to be non-existent.

Of the vitamin A₁ isomers to which reference has been made all-*trans* and 13-*cis* vitamin A₁ occur f. inst. in fish liver oils, while 11-*cis* vitamin A₁ has been found in the eyes of lobster (WALD and BURG 1955) and in *Meganyctiphanes norvegica* and *Thysanoessa raschii* presumably together with small quantities of 11,13-di-*cis* vitamin A₁, and in various species of *Pandalus*, *P. bonnieri*, *P. borealis* and *P. montagui* (FISHER, HENRY, KON, PLACK and THOMPSON 1955, PLACK, FISHER, HENRY and KON 1956). The u. v. absorption curve of the vitamin A₁ found by LAMBERTSEN and BRÆKKAN (1955) in the eyes of *Pandalus borealis* is displaced towards lower wavelengths as compared to the curve of all-*trans* vitamin A₁. Basing their opinion on the u. v. absorption curve and the maleic anhydride reaction LAMBERTSEN and BRÆKKAN assume this displacement to be due to the presence of 9,13-di-*cis* vitamin A₁.

The experiments which have hitherto been made with the object of ascertaining the presence of the different *cis* isomers in natural vitamin A₁ have depended either on the course of the u. v. absorption curve or, mainly, on the reaction between vitamin A₁ and maleic anhydride. It is well known that maleic anhydride interacts with the two terminal double bonds in the vitamin A₁ side chain to form an addition product which does not yield a blue colour with antimony trichloride (ROBESON et al. 1955). The rate of this reaction is considerably greater in the case of the isomers with *trans* configuration at the double bonds No. 11 and No. 13, than in the case of molecules with *cis* configuration at either or both of these double bonds. Incidentally, the rates of reaction ascertained in the case of the individual 'slow' reacting vitamin A₁ isomers differ too.

By following the course of the maleic anhydride reaction as done by ROBESON and BAXTER (1947) it is possible to determine the relative proportions of 13-*cis* and all-*trans* vitamin A₁ in a mixture, if none of the other isomers are present or, more generally (PLACK 1956), the relative proportions of 'fast' and 'slow' reacting components in an arbitrary mixture of vitamin A₁ isomers. In such a mixture of an unknown number of vitamin A₁ isomers the

method does not, however, allow for any determination of the individual isomer or isomers represented in the two groups in the mixture.

It has already been shown (BRO-RASMUSSEN, HJARDE and POROTNIKOFF 1955, BARNHOLDT and HJARDE 1956) that vitamin A isomers can be separated by chromatography.

In the following a description will be given of an investigation on the content of vitamin A in the eyes of *Pandalus borealis* by fractional chromatography with a special view to the determination of the configuration of and quantity in which the individual vitamin A isomers present occur.

Experimental.

The light petroleum, diethyl ether and dicalcium phosphate used for the chromatography comply with the requirements described in a previous publication (BRO-RASMUSSEN, HJARDE and POROTNIKOFF 1955). Saponification and extraction were performed as previously described (HJARDE 1950). Saponification, extraction and chromatography as well as all subsequent experiments were performed in rooms (temperature 22–23° C) protected from daylight.

Ultraviolet absorption curves were determined by means of a Beckman quartz spectrophotometer, model DU, with 1 cm cells. The Carr-Price measurements were also made by means of this instrument.

The prawns were bought in cooked condition and had not been protected from daylight during transport to the laboratory and removal of the eyes.

I. Chromatography of the Unaponifiable Fraction from the Eyes of Pandalus borealis.

1, 1. Experiment 1.

The unaponifiable fraction from 43 g of prawns' eyes (including the tissue immediately surrounding the eyes) derived from 5 kg of prawns was dissolved in light petroleum and subjected to a preliminary chromatographic purification on a 17×1.8 cm column of alumina (Merck Al_2O_3 , standardized according to Brockman, deactivated by the addition of 4 % H_2O). Elution was performed with a mixture of diethyl ether and light petroleum (diethyl ether concentrations varying from 20 to 60 % v/v). The vitamin A isolated in this way exhibited when dissolved in light petroleum a curve with a broad peak extending from 320 to 325 $\text{m}\mu$. $E_{310}/E_{325} = 0.945$, $E_{330}/E_{325} = 0.431$. $E_{1\text{cm}, 325\text{m}\mu}^{\%} = 0.137$ calculated on the basis of the weight of eyes used.

The resulting solution of vitamin A in light petroleum was next chromatographed on a 47×3.0 cm dicalcium phosphate column,

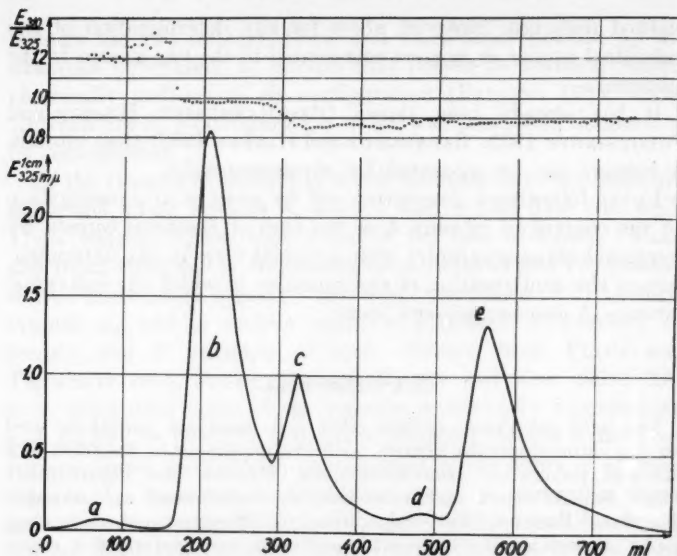


Fig. 1. Fractionation curve from the chromatography of the unsaponifiable fraction of the eyes of *Pandalus borealis* (3 kg of prawns) on a 78×3.0 cm column of dicalcium phosphate. Collection of eluate in 5 ml fractions. The upper part of the figure illustrates the value of the ratio E_{310}/E_{325} measured directly on the collected fractions.

elution being performed with mixtures of diethyl ether and light petroleum in which the diethyl ether concentration varied from 3 to 7 % v/v. Eluate was collected first in 10 ml fractions and subsequently in 25 ml fractions.

I, 2. Experiment 2.

Correspondingly, the unsaponifiable fraction from 28.5 g of eyes derived from 3 kg of prawns was subjected to fractional chromatography on a 78×3.0 cm column of dicalcium phosphate. Eluate was collected in 5 ml fractions. Fig. 1 shows the fractionation curve. Better separation has been obtained in this case than when using the column dimensions mentioned under I, 1. A comparison of the course of the two fractionation curves and of the variation in the ratios E_{310}/E_{325} and E_{350}/E_{325} throughout the series of fractions collected in the two chromatographic separations, however, supports the assumption that in both cases we have to do with the same five substances, in the following denoted a, b, c, d, and e, mentioned in the order in which they are eluted.

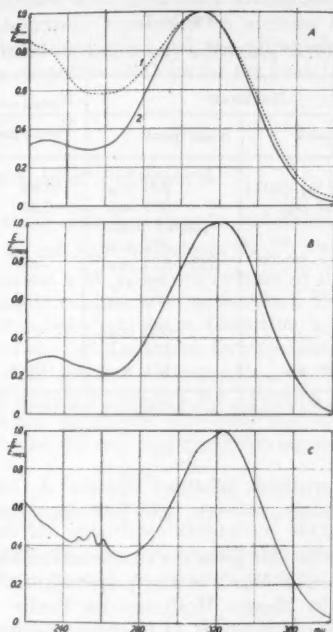


Fig. 2. U. v. absorption curves measured in absolute ethanol.

- A: Curve 1: u. v. absorption curve for substance *a*. Curve 2: u. v. absorption curve for the F. G. isomer.
 B: U. v. absorption curve for substance *b*.
 C: U. v. absorption curve for substance *d*. The three small peaks at about 250–260 $m\mu$ are presumably due to irrelevant substances with u. v. absorption.

Expressed by $E_{325\ m\mu}^{1\text{cm}}$ measured directly on the eluted fractions the relative proportions of the substances present were found to be as follows:

Experiment I, 1: $a:b:c:e = 0.8:52:9:38$ (this chromatogram gave a very incomplete separation of substance *d* from substances *c* and *e*; the contribution yielded by substance *d* towards the total extinction at 325 $m\mu$ of the collected fractions was estimated at 0.5–1 %).

Experiment I, 2: $a:b:c:d:e = 1.5:49:17:1.5:31$.

After evaporation at room temperature in a stream of CO₂, peak fractions with maximum contents of each of the five substances *a*, *b*, *c*, *d*, and *e* from I, 2 were taken up in absolute ethanol. A survey of the spectrophotometric properties of the five substances is given in table I, which also lists the corresponding properties of all-*trans* and 13-*cis* vitamin A₁ prepared in this laboratory by chromatography of

Table 1.
Spectrophotometric properties (measured in absolute ethanol).

Substance	Maximum		$E_{\text{small peak}}$	E_{310}	E_{350}
	small peak	main peak	$E_{\text{main peak}}$	E_{325}	E_{325}
<i>a</i>	abt. 228 $m\mu$ (infl.)	310 $m\mu$	0.82	1.15	0.38
<i>b</i>	234 $m\mu$	321 $m\mu$ (broad max.)	0.30	0.95	0.43
<i>c</i>	—	327 $m\mu$	—	0.85	0.51
<i>d</i>	—	323–324 $m\mu$	—	0.88	0.46
<i>e</i>	—	325 $m\mu$	—	0.88	0.45
all- <i>trans</i>					
vit. A_1	—	325 $m\mu$	—	0.856	0.449
13- <i>cis</i> vit. A_1	—	328 $m\mu$	—	0.809	0.543
F. G. -isomer	229 $m\mu$	311 $m\mu$	0.340	1.18	0.329

¹ The location of 13-*cis* vitamin A_1 's subsidiary maximum has not been ascertained.

iodine-isomerized, synthetic all-*trans* vitamin A_1 (ordinary artificial light, room temperature, solvent: light petroleum, period of action of iodine: 30 seconds).

In addition the table also gives the characteristic absorption maxima of a vitamin A_1 isomer found in the preparation "Vitamin A, Feed Grade", marketed by Messrs. Hoffmann-La Roche & Co., Basle. It is assumed (cf. p. 62), that this is 11,13-di-*cis* vitamin A_1 . In the following this isomer is denoted the F. G. -isomer. The ultraviolet absorption curves of this substance and substance *a* are given in Fig. 2 (A).

I, 3. Rechromatography of Substance b.

Ultraviolet absorption curves measured in absolute ethanol for the peak fractions corresponding to substance *b* from the two dicalcium phosphate chromatograms described in section I, 1 and I, 2 showed good agreement within the wavelength range 290–400 $m\mu$, while within the range 220–290 $m\mu$ the value of E/E_{max} for substance *b* isolated in the course of experiment 1 (I, 1) exceeded by 0–0.060 the value measured for substance *b* isolated in the course of experiment 2 (I, 2). The peak fractions of *b* from I, 1 ($E_{325}^{1 \text{ cm}} = \text{appr. } 25$ in 10 ml light petroleum) was rechromatographed on 100 \times 1.3 cm fine-grained alumina weakened by being mixed with 7 % by weight of water (BARNHOLDT 1956). Elution with 5–18 % v/v diethyl ether in light petroleum. The course of the fractionation curve indicated that only one substance with absorption within the range 310–350 $m\mu$ was present, and the peak fractions dissolved in absolute ethanol gave an absorption curve which was identical with that found in experiment I, 2 for substance *b* within the range 220–400 $m\mu$. Further chromatography on a 100 \times 1.8 cm column of dicalcium phosphate (washed with absolute ethanol to

remove any impurities with ultraviolet absorption) of peak fractions from the chromatographic separation on alumina again indicated the presence of one substance only; and the ultraviolet absorption curve (Fig. 2 (B)) in the range 220—400 $m\mu$ for the peak fraction was still the same.

I, 4. Rechromatography of Substance d.

The peak fractions corresponding to substance *d* (experiment 2) were collected in 10 ml light petroleum ($E_{325}^{1\text{ cm}} = 0.50$) and chromatographed again on a 48×1.0 cm column of dicalcium phosphate (washed with absolute ethanol after activation). Elution with 3—7 % (v/v) diethyl ether in light petroleum. Collection in 3 ml fractions. The fractionation curve showed 3 maxima corresponding to the substances *c*, *d* and *e*. The peak fraction corresponding to substance *d* showed, when dissolved in absolute ethanol, the following spectrophotometric properties: One main maximum: 323—324 $m\mu$ and three subsidiary maxima: 249, 255 and 261 $m\mu$ and the following ratios:

$$\frac{E_{310}}{E_{324}} = 0.914$$

$$\frac{E_{350}}{E_{325}} = 0.447$$

$$\frac{E_{324}}{E_{325}} = 1.007$$

Fig. 2 (C) shows the ultraviolet absorption curve. The three small peaks at about 250—260 $m\mu$ are presumably caused by impurities, which it has not been possible to separate from the small quantities of substance *d*.

II. Identification.

II, 1. Isomerization.

The five substances *a*, *b*, *c*, *d*, and *e* were dissolved in *n*-hexane and isomerized by the addition of iodine. A solution (5 ml) of the substance in question with $E_{\text{max}}^{1\text{ cm}} = \text{about } 0.5$ was mixed with 0.5 ml *n*-hexane containing altogether 0.05 mg iodine. After shaking for 30 seconds at room temperature in ordinary artificial light, 0.5 g of pulverized sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) was added. When the iodine colour, after shaking for a short time, had disappeared, the solution was filtered, and the isomerate was subjected to spectrophotometric measurement. In all five cases this gave $\lambda_{\text{max.}} = 325\text{ }m\mu$ and curves the course of which may be characterized by reference to the data given in table 2 (measured in *n*-hexane). Incidentally, the iodine treatment does not only result in isomerization, but gives rise to certain secondary reactions, when effected under the conditions described. If the duration of the action of iodine is extended so as to last for several minutes, the value of ratio E_{350}/E_{325} will increase, assuming values exceeding 0.5.

Table 2.

Spectrophotometric properties before and after isomerization.
(in n-hexane solution).

	Maximum, m μ		$\frac{E_{316}}{E_{325}}$		$\frac{E_{350}}{E_{325}}$		$\frac{E_{\max. \text{ after}}}{E_{\max. \text{ before}}}$
	before	after	before	after	before	after	
<i>a</i>	308—09	325	1.21	0.86	0.33	0.47	1.42
<i>b</i>	320—23	325	0.94	0.85	0.42	0.47	1.38
<i>c</i>	327	325	0.85	0.85	0.48	0.47	1.08
<i>d</i>	323—24	325	0.90	0.88	0.43	0.50	abt. 1.0
<i>e</i>	325	325	0.86	0.86	0.42	0.47	0.95
all- <i>trans</i>							
vit. A ₁	325	325	0.85	0.86	0.46	0.48	0.88
13- <i>cis</i>							
vit. A ₁	328	325	0.80	0.85	0.54	0.48	0.95
F. G. isomer	308—09	325	1.23	0.86	0.31	0.48	1.50

Table 2 also gives the results obtained by isomerization, performed under identical conditions, of all-*trans*, 13-*cis* vitamin A₁ and the F. G. isomer. (Same preparations as those previously mentioned — confer I, 2.)

II, 2. Formation of anhydro vitamin.

Substances *a*, *b*, *c*, and *e* were dissolved in absolute ethanol and treated with dry ethanolic hydrogen chloride, 0.20 N, temperature 25—26° C, as described elsewhere (BARNHOLDT 1957). The small quantities of substance *d* available did not allow for the use of this identification test.

For purposes of comparison 13-*cis* and all-*trans* vitamin A₁ as well as the F. G. isomer have also been included in this examination.

Curves representing $E_{390}/E_{\max.}^{\circ}$ (refer the above work) plotted against time from the moment of admixture are given in Fig. 3.

On treatment with ethanolic hydrogen chloride solution the reaction mixtures obtained from all of the substances examined exhibited maxima at 349, 368, and 390 m μ , presumably because in all cases the same substance, anhydro vitamin A₁, was formed. However, this problem has not been examined further in the present work. ROBESON and BAXTER (1947) have shown that both 13-*cis* and all-*trans* vitamin A₁ on treatment with hydrogen chloride form anhydro vitamin A₁.

II, 3. The ratio: $\frac{E_{\text{Carr-Price}}}{E_{\text{u.v. max.}}}$

Substances *a*, *b*, and *d* as well as 13-*cis* and all-*trans* vitamin A₁ and the F. G. isomer were all subjected to the Carr-Price test as follows: 1 ml of a solution in chloroform of the substance in question (the

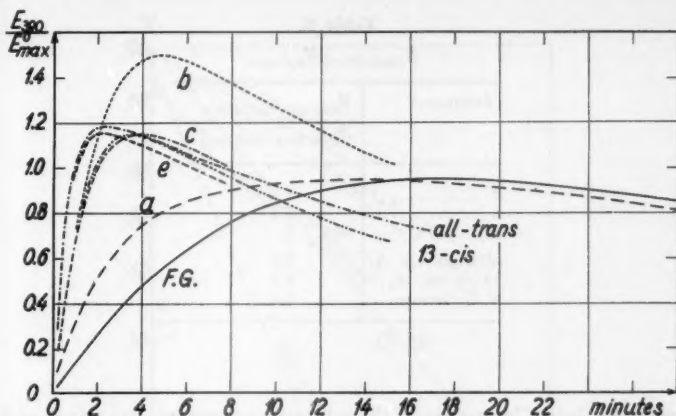


Fig. 3. Formation of anhydro vitamin A₁ in 0.20 N anhydrous hydrogen chloride in absolute ethanol. The curves represent E_{390}/E_{0max} for each of the substances a, b, c, e, the F. G. isomer, all-trans and 13-cis vit. A₁ plotted against time reckoned from the moment of mixing hydrogen chloride solution and test substance. The maxima are characterized by the following values for $t_{max.anh.}$ and $\left(\frac{E_{390}}{E_{0max}}\right)_{max}$.

(cf. BARNHOLT, 1957):

a: 14–15 min., 0.948. b: 4.8 min., 1.50. c: 4.0 min., 1.14.

e: 2.2 min., 1.14. all-trans vit. A₁: 2.0 min., 1.18. 13-cis vit. A₁: 3.5 min., 1.14. F. G. isomer: 17–18 min., 0.950.

concentration being determined in advance by measuring $E_{u.v.}^{l cm}$ (= appr. 0.5) of the substance in an ethanolic solution of the same concentration as that of the chloroform solution) was mixed with 2 ml of a saturated solution of antimony trichloride in chloroform (containing 2.5 % v/v acetic acid anhydride), and $E_{620 m\mu}^{l cm}$ was measured six seconds after the admixture (Beckman model DU, slit width 0.05 mm). The figures given in table 3 are mean figures resulting from several determinations of the ratio $\frac{E_{620 C.P.}}{E_{u.v.}^{l cm}}$, except in the case of substance d. The small quantity in which this substance was available allowed for a single measurement only.

II, 4. The maleic anhydride reaction.

The course of the reaction between maleic anhydride and each of the substances a, b, and d and (for purposes of comparison) the F. G. isomer, all-trans and 13-cis vitamin A₁ was examined in the following

Table 3.

$$E_{\text{Carr-Price}}/E_{\text{u.v. max}}$$

Substance	$E_{\text{Carr-Price}}/E_{\text{u.v. max}}$	
	$E_{620 \text{ m}\mu, \text{ Carr-Price}}$	$E_{\text{u.v. max. (ethanol)}}$
<i>a</i>	4.2	
<i>b</i>	4.1	
<i>d</i>	abt. 3	
all- <i>trans</i> vit. A ₁	2.6	
13- <i>cis</i> vit. A ₁ ..	2.8	
F. G. isomer...	4.4	

way. A solution in benzene of the substance in question was mixed with the same volume of a solution of maleic anhydride in benzene (prepared by dissolving 10 g maleic anhydride in 100 ml benzene). The quantity of non-converted vitamin A was determined immediately after the admixture and at suitable intervals (in the case of all-*trans* vitamin A₁ at intervals of a few minutes, in the case of the other substances at intervals of 24–48 hours) by withdrawing 0.1 ml of the reaction mixture, adding 0.9 ml chloroform + 2 ml of a saturated solution of antimony trichloride in chloroform (to which 2.5 % v/v acetic acid anhydride had been added immediately before use) and measuring the blue colour at 620 m μ after an interval of 6 seconds. Between measurements the reaction mixture was set aside in the dark at 22–23° C. For all of the substances examined (except *d*) the concentration of the benzene solution was such that $E_{620 \text{ m}\mu}^{1 \text{ cm}}$ in the Carr-Price reaction immediately after the addition of maleic anhydride was found to be about 0.6–0.7 — in the case of substance *d* it was only about 0.1, on account of the small quantity in which this substance was available.

The Carr-Price reaction performed on solutions in pure benzene of all-*trans* vitamin A₁, 13-*cis* vitamin A₁, the F. G. isomer and substance *b*, which had been set aside for the same periods and under the same conditions as the maleic anhydride reaction mixtures, did not show any loss of vitamin A to occur during periods of this duration.

Fig. 4 illustrates the vitamin A concentration to a logarithmic scale plotted against time (the initial concentration being designated as 100). From each of these curves the velocity constant *k* in the equation $\ln \frac{c}{c_0} = -kt$ has been calculated (*c*₀ being the concentration of vitamin A₁ at the time 0, *c* being the concentration at the time *t*).

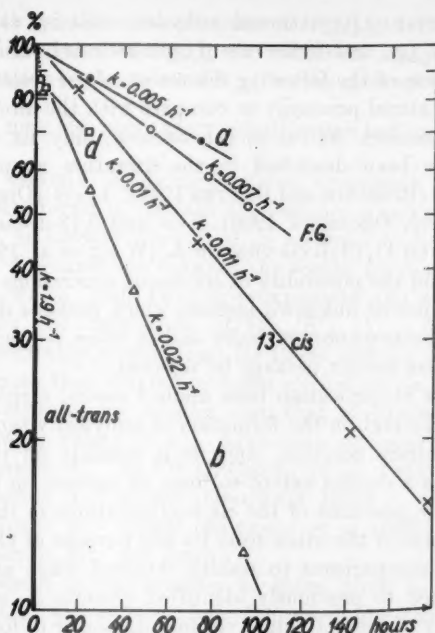


Fig. 4. Maleic anhydride reaction in benzene solution (5 g per 100 ml) at 22–23° C. The course of the reaction between maleic anhydride and each of the substances *a*, *b*, *d*, the F. G. isomer, all-*trans* and 13-*cis* vit. A₁ was followed by measurements of $E_{620 \text{ m}\mu}^{\text{1cm}}$ by the Carr-Price reaction. k is the velocity constant in the equation

$$\ln \frac{c}{c_0} = -kt.$$

Discussion.

Experiments I, 1 and I, 2 show that chromatographic separation of the unsaponifiable fraction of the eyes of deep-water prawns demonstrates the presence of five substances, designated *a*, *b*, *c*, *d*, and *e*, which must be assumed to be vitamin A₁ isomers. The isomerization with iodine as catalyst described in section II, 1 confirms this assumption, since the isomerization product of all five substances show an u. v. absorption maximum (in n-hexane) at 325 m μ . The object of the subsequent investigations has been to identify the individual substances. Identification tests applied to the different fractions were: (1) the u. v. absorption curve, (2) alterations in this curve on isomerization with iodine, (3) altera-

tions in the curve on formation of anhydro vitamin, (4) the ratio $E_{\text{Carr-Price}}/E_{\text{u.v.,max.}}$, and (5) the rate of the maleic anhydride reaction.

In the course of the following discussion of the results obtained it has been natural primarily to compare with the already known vitamin A_1 isomers. As far as can be seen, only six vitamin A_1 isomers have been described in the literature at present: all-*trans*, 13-*cis* (ROBESON and BAXTER 1947), 11-*cis* (DIETERLE and ROBESON 1955, OROSHNIK 1956), 9-*cis* and 9,13-di-*cis* (ROBESON et al. 1955) and 11,13-di-*cis* vitamin A_1 (WALD et al. 1955, OROSHNIK 1956), and the possibility of erroneous conclusions on account of the existence of unknown isomers which perhaps deviate very little both spectrophotometrically and in other respects from the known isomers cannot perhaps be rejected.

Among the identification tests applied special emphasis should presumably be laid on the formation of anhydro vitamin and the maleic anhydride reaction, since it is possible on the basis of these tests to a certain extent to form an opinion on the number as well as the positions of the *cis* configurations in the molecule, while the value of the other tests for the purpose of identification depends on comparisons to results obtained when applying the test concerned to previously identified vitamin A_1 isomers.

The ability of the individual vitamin A_1 isomer to form anhydro vitamin must be assumed to depend on the number of *cis* configurations in the molecule (confer BARNHOLDT 1957), since the presence of *cis* configurations delays the formation of anhydro vitamin A_1 . As already shown by ROBESON and BAXTER (1947) a *cis* configuration at one double bond will increase the time required to obtain maximum anhydro vitamin A_1 concentration ($t_{\text{max.anh.}}$) as compared to $t_{\text{max.anh.}}$ for all-*trans* vitamin A_1 . In the case of isomers with *cis* configuration at two or more double bonds a further increase of this period might be anticipated. As appears from Fig. 3, substances *a*, *b*, *c*, and *e* can be classified according to the measured values of $t_{\text{max.anh.}}$ in three groups. Substance *e* with a period of about 2 min., corresponding to all-*trans* vitamin A_1 , substances *b* and *c* with the period 4–5 min., corresponding to *neo a* vitamin A_1 , and substance *a* with the period 14–15 min. The F. G. isomer exhibited a $t_{\text{max.anh.}}$ value almost like that of substance *a*. Thus substance *e* seems to be all-*trans* vitamin A_1 , while substances *b* and *c* have *cis* configuration at one double bond and substance *a* and the F. G. isomer at two or more double bonds.

In addition to the measurements of $t_{\max, \text{anh.}}$, determination of the ratio of the value of $E_{390 \text{ m}\mu}$ at maximum anhydro vitamin A₁ concentration to the value of $E_{\max.}$ at the moment of admixture

$\left(\frac{E_{390}}{E_{\max.}^0} \right)_{\max.}$ may also serve as an identification test in comparisons

to known vitamin A₁ isomers. Good agreement between the two ratios was found in the case of substance *a* and the F. G. isomer, which, as already mentioned, is assumed to be 11,13-di-*cis* vitamin A₁. A comparison between the results for *b* and for 13-*cis* vitamin A₁, which both have approximately the same $t_{\max, \text{anh.}}$ value, shows the magnitude of $(E_{390}/E_{\max.}^0)_{\max.}$ for substance *b* to be considerably greater than for 13-*cis* vitamin A₁.

This suggests that maximum molar extinction ($\epsilon_{\max.}$) of substance *b* is considerably smaller than that of 13-*cis* vitamin A₁.

The following values of $\epsilon_{\max.}$ have been reported:

13-*cis* vitamin A₁: 48,300 (ROBESON et al. 1955)

11-*cis* vitamin A₁: 32,500 (OROSHNIK 1956)

9-*cis* vitamin A₁: 42,300 (ROBESON et al. 1955)

7-*cis* vitamin A₁: not known

A comparison of these values to those given in table 3 for $(E_{390}/E_{\max.}^0)_{\max.}$ seems to exclude the possibility that substance *b* is 9-*cis* vitamin A₁, while it appears probable that it is identical with 11-*cis* vitamin A₁.

In the case of substance *c* the value of both $\left(\frac{E_{390}}{E_{\max.}^0} \right)_{\max.}$ and $t_{\max, \text{anh.}}$ show fairly good agreement with those found for 13-*cis* vitamin A₁. In the case of substance *e* both $\left(\frac{E_{390}}{E_{\max.}^0} \right)_{\max.}$ and $t_{\max, \text{anh.}}$ show good agreement with the values found for all-*trans* vitamin A₁.

The value of the maleic anhydride reaction lies in the fact that it enables a determination of the position of the *cis* configurations. As mentioned above, maleic anhydride interacts with double bonds in positions 11 and 13, for which reason a *cis* configuration in either or both of these positions will delay the reaction. By means of the maleic anhydride reaction it is thus possible to ascertain in the case of either of these isomers whether they have 11,13-di-*trans* configuration. Of the isomers examined (Fig. 4) *a*, *b*, and *d* are all seen to react slowly with maleic acid anhydride. These three isomers thus have a *cis* configuration at not less than

one of the two terminal double bonds. In the case of substance *a* the rate of reaction is somewhat lower than in the case of *b* and *d*, which perhaps suggests a *cis* configuration at both double bonds.

As shown by Fig. 4, the rate of reaction in the case of the F. G. isomer ($k = 0.007 \text{ h}^{-1}$) is of the same magnitude as in the case of substance *a* (0.005 h^{-1}). OROSHNIK (1956) found for 11,13-di-*cis* vitamin A_1 : $k = 0.0054 \text{ h}^{-1}$ under experimental conditions which differed from those applied in the present examination. It should be borne in mind that the inaccuracy of the measurements in the case of substance *a* is fairly great on account of the small quantity of the substance available.

In the following the results of the identification tests will be summarized for each of the isomers.

The F. G. isomer: The measurements in connection with the formation of anhydro vitamin A_1 and the rate of reaction with maleic anhydride suggest that this isomer has *cis* configuration at both the double bonds in positions 11 and 13. The u. v. absorption curve (Fig. 2 (A)) shows a characteristic resemblance to the curve plotted by WALD et al. (1955) for the "311 $m\mu$ isomer", which, by OROSHNIK (1956), is stated to be 11,13-di-*cis* vitamin A_1 . WALD et al. thus found in ethanolic solution $\lambda_{\text{max.}} = 311 \text{ m}\mu$ (the F. G. isomer also gives $\lambda_{\text{max.}} = 311 \text{ m}\mu$), in hexane solution $\lambda_{\text{max.}} = 310 \text{ m}\mu$ (the F. G. isomer 309 $m\mu$). The curve given by WALD et al. shows a subsidiary maximum at about 229 $m\mu$ for which the ratio E_{220}/E_{311} is found to be 0.29. A corresponding maximum was found in the case of the F. G. isomer, although in this case $E_{220}/E_{311} = 0.34$.

On the basis of these results the F. G. isomer may presumably be stated to be 11,13-di-*cis* vitamin A_1 .

Substance a shows an u. v. absorption curve which with respect to wavelengths above 300 $m\mu$ has practically the same course as that of the F. G. isomer. At wavelengths below 300 $m\mu$ the location of the curve for *a* is above that of the F. G. isomer — presumably on account of impurities with u. v. absorption in the fractions isolated by dicalcium phosphate chromatography. Attempts at further chromatographic purification of substance *a* which was only available in fairly small quantities have not been made. The subsidiary maximum at 229 $m\mu$ found for the F. G. isomer and by WALD et al. for their 311 $m\mu$ isomer is, on account of the presence of these impurities, masked in the case of the curve for

substance *a*, although an inflexion is seen at about 228 mμ which may be taken to correspond to this maximum.

The remaining identification tests show relatively small differences between substance *a* and the F. G. isomer which may be explained by the presence of irrelevant substances in the collected fractions of substance *a* (possibly, f. inst. small quantities of substances *b*, *c*, *d*, and *e*), which have not been separated out completely in the chromatography. The course of the process of formation of anhydro vitamin suggests that this is the case.

The value of the ratio $\frac{E_{\text{Carr-Price}}}{E_{\text{u.v. max.}}}$ for substance *a* is of the

same magnitude as for the F. G. isomer. It is considerably higher than the corresponding value for 13-*cis* and all-*trans* vitamin A₁, a finding which supports the assumption that substance *a* and the F. G. isomer contain a hindered *cis* configuration. On isomerization an increase in the extinction at the maximum in the u. v. absorption curve is observed, which shows *a* to have a lower molar extinction coefficient than the conversion product: the mixture of all-*trans* and 13-*cis* vitamin A₁ obtaining at equilibrium. This is in good agreement with findings obtained as regards the formation of anhydro vitamin as well as measurements of the ratio $\frac{E_{\text{Carr-Price}}}{E_{\text{u.v. max.}}}$.

On the basis of the properties described it seems reasonably safe to conclude that substance *a* is also identical with the "311 mμ isomer" prepared by WALD et al. (1955), the substance which according to OROSHNIK (1956) is 11,13-di-*cis* vitamin A₁.

Substance *b*. In absolute ethanol this substance shows an u. v. absorption maximum at 321 mμ (broad maximum), i. e. the same wavelength as the one given for the maximum displayed by 11-mono-*cis* (*neo b*) vitamin A₁ (OROSHNİK 1956, WALD et al. 1955). The curve published by WALD et al. for *neo b* vitamin A₁ shows a subsidiary maximum at about 230 mμ, and the ratio E_{230}/E_{321} can be found to be 0.29. DIETERLE and ROBESON (1954) give the data: subsidiary maximum at 233 mμ, main peak at 322 mμ and $\frac{E_{233}}{E_{322}} = 0.29$. In the case of substance *b* (table 1) the findings were:

subsidiary maximum at 234 mμ and $\frac{E_{334}}{E_{321}} = 0.30$. Apart from the discrepancy with regard to the position of the subsidiary max-

imum as given by WALD et al., there is thus good agreement with the absorption curve found for substance *b* and the data given by WALD et al. as well as by DIETERLE and ROBESON. The increase in the E_{\max} value on iodine isomerization (table 2) which, compared to the values applying in the case of 13-*cis* and all-*trans* vitamin A_1 , is fairly large also suggests that substance *b* contains a hindered *cis* configuration, an assumption which is further supported by the maximum value of $\frac{E_{330}^o}{E_{321}^o}$ ($= 1.50$)

obtained in the formation of anhydro vitamin and of the magnitude of the ratio $\frac{E_{\text{Carr-Price}}}{E_{\text{u.v.,max.}}} (= 4.1)$. A comparison between the *k*

value $= 0.022 \text{ h}^{-1}$ found for the maleic anhydride reaction with substance *b* and the value $k = 0.03 \text{ h}^{-1}$ given by OROSHNIK (1956) for 11-*cis* vitamin A_1 does not show full agreement, but this may be due to differences in experimental conditions. The technique used by OROSHNIK has not been described in details. Otherwise the maleic anhydride reaction, as well as the measurement of $t_{\max, \text{anh.}}$ suggests that substance *b* is either 11- or 13-mono-*cis* vitamin A_1 . Since 13-mono-*cis* vitamin A_1 is already known under the name of *neo a* vitamin A_1 , these results may also be taken to indicate that substance *b* is 11-*cis* vitamin A_1 .

Substance c. A comparison between the spectrophotometric properties of this substance and those of the *neo a* vitamin A_1 preparations seems to show, with certainty, that we have to do with 13-mono-*cis* vitamin A_1 (*neo a*). The identity is further confirmed by the remaining tests made (isomerization, formation of anhydro vitamin). However, the spectrophotometric measurements (table 1) as well as the course of the formation of anhydro vitamin suggest that the 13-*cis* vitamin A_1 (substance *c*) isolated here contains slight impurities consisting of substance *b*.

Substance d. For this substance λ_{\max} was found to be 323–324 $m\mu$ (in ethanol), a finding which prompts a comparison to the substances 9-mono-*cis* and 9,13-di-*cis* vitamin A_1 , since λ_{\max} for these substances (in ethanol) are stated (ROBESON et al. 1955) to be 323 and 324 $m\mu$, respectively. According to ROBESON et al. (1955) the u. v. absorption curves for 9-*cis* and 9,13-di-*cis* vitamin A_1 display subsidiary maxima at about 260 $m\mu$. As the u. v. absorption curve of the substance *d* isolated in the present investigation (Fig. 2 (C)) suggests the presence of an irrelevant

substance with absorption about 260 mμ, it is not possible to establish the location of a possible subsidiary maximum corresponding to substance *d*.

As mentioned in the above, the maleic anhydride reaction shows the substance to have *cis* configuration at not less than one of the terminal double bonds, for which reason the possibility that it is 9-mono-*cis* vitamin A₁ can be disregarded. The rate of the maleic anhydride reaction, determined, however, on the basis of very few measurements on account of the small quantity of substance available, is approximately of the same magnitude as the rate found for 13-*cis* vitamin A₁ under corresponding conditions.

The value of the ratio $\frac{E_{\text{Carr-Price}}}{E_{324}} = \text{appr. } 3$ and the fact

that E_{max} did not change on isomerization seem to suggest that substance *d* does not contain any hindered *cis* configuration. However, these results should be viewed with a certain reservation, considering *f. inst.* that even rechromatography of substance *d* did not result in any complete separation from substances *c* and *e*.

According to ROBESON et al. (1955) 9,13-di-*cis* vitamin A₁ (in the form of the *p*-phenylazobenzoate ester dissolved in benzene) may be isomerized with iodine in the dark to form 9-mono-*cis* vitamin A₁ (68.5 %) + 9,13-di-*cis* vitamin A₁ (31.5 %), *i. e.* a mixture the maximum of which is below 325 mμ. By the isomerization performed in the course of the present investigation in an artificially illuminated room an isomerization product with $\lambda_{\text{max}} = 325 \text{ m}\mu$ was obtained, which suggests that the isomerization has resulted in the formation of the previously mentioned equilibrium mixture of all-*trans* and 13-*cis* vitamin A₁. Since as already mentioned the conditions under which the isomerization has been performed deviate from those applied by ROBESON et al. (1955), this observation can hardly be considered to disprove the assumption that substance *d* is identical with 9,13-di-*cis* vitamin A₁, but further investigations are required to fully establish the identity of substance *d*.

Substance e. The spectrophotometric properties of substance *e* as well as its location in the dicalcium phosphate column in relation to the other vitamin A₁ isomers prompts a comparison with all-*trans* vitamin A₁. Even if the increase in E_{max} on iodine isomerization is slightly greater in the case of substance *e* than in the case of all-*trans* vitamin A₁ and although $t_{\text{max.anh.}}$ was found

to be 2.2 minutes instead of 2.0 minutes as found for all-*trans* vitamin A₁, it seems preponderantly probable that substance *e* is identical with all-*trans* vitamin A₁. The discrepancies mentioned are presumably due to the presence in the collected fractions corresponding to substance *e* of small quantities of the substances eluted prior to *e*.

Summary.

In fractional chromatography of the unsaponifiable fraction from the eyes of deep-water prawns the vitamin A₁ present was found to separate into five components: *a*, *b*, *c*, *d*, and *e*. In two instances the relative proportions of the different isomers present, expressed by $E_{325\text{ m}\mu}$ measured on the collected fractions were found by chromatography to be as follows:

1. $a:b:c:e = 0.8:52:9:38$ (*d* was incompletely separated from *c* and *e*, the quantity of *d* may be estimated to be about 0.5—1 %).
2. $a:b:c:d:e = 1.5:49:17:1.5:31$.

The substances obtained were subjected to a number of identification tests: isomerization with iodine as catalyst, formation of anhydro vitamin by means of hydrogen chloride, determination of $E_{620\text{ m}\mu}$, Carr-Price/ $E_{\text{u.v. max.}}$ and the maleic anhydride reaction. The spectrophotometric properties of the substances have also been examined for the purpose of establishing their identity.

The results obtained suggest that substances *a*, *b*, *c*, and *e* are identical with 11,13-di-*cis*, 11-mono-*cis*, 13-mono-*cis* and all-*trans* vitamin A₁, respectively, while the small quantities of substance *d* available have rendered the identification of this substance less certain. The possibility that substance *d* is 9,13-di-*cis* vitamin A₁ is discussed. In a preparation marketed by Messrs. Hoffmann-La Roche & Co., Basle, under the name of "Vitamin A, Feed Grade", the presence has been ascertained of an isomer the properties of which agree with those of substance *a* and 11,13-di-*cis* vitamin A₁.

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Cardiovascular Effects of Direct Vagal Stimulation in Man.

By

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It is known from studies in animals that direct stimulation of the peripheral ends of the cut vagal nerves has remarkable inhibitory effects on the heart rate, even at very low stimulation rates. The correlation between stimulation frequency and effector response forms a hyperbolic curve, where maximal effects are usually reached at 10 to 20 impulses per second, which is typical of most autonomic neuroeffectors (see ROSENBLUETH 1950 and FOLKOW 1955). — It is generally agreed that the ventricular myocardium is not directly influenced by vagal stimulation in mammals. It has recently been observed, however, that in man a prompt and often marked reflex decrease of stroke volume may occur on stimulation of the carotid sinus region, which has aroused the question whether in this species vagal fibres directly affect also the contractility of the heart ventricles (PETERSON 1951).

In patients operated on for cancer in the neck it is a routine procedure to dissect free the vagal nerves and the big vessels and it has sometimes been observed that under such circumstances complications can follow, for instance a sudden drastic fall in blood pressure. The present experiments were started in order to analyse these cardiovascular changes and, if possible, try to eliminate them. In the course of the study it was in some cases possible to stimulate the vagal nerves electrically, which made it

possible to analyse certain aspects of the parasympathetic control of the heart function in man.

Further, it is known from animal experiments that especially thin afferent nerve fibres are easily activated by mechanical stimuli. It was therefore probable that the unavoidable traction on the vagal nerves may cause a mechanical stimulation of its different types of fibres. The cardiovascular effects of the dissection procedures were therefore followed continuously throughout the operations and attempts were made to analyse the nature of induced changes.

Method.

A combination of ether, nitrous oxide and Evipan was used as anesthesia. From the start of the operation the blood pressure, the pulse rate and the pulse amplitude were recorded directly by means of a continuous intra-arterial blood-pressure measurement from an indwelling polyethylene catheter, introduced percutaneously into the brachial artery (BERNEUS, CARLSTEN and HOLMGREN 1954). The catheter was connected to a strain-gauge manometer (Elema), operating a sixchannel electrocardiograph (Mingograph, Elema), which shows a practically linear correlation between amplitude and pressure load. The electrocardiogram was recorded concomitantly to allow an analysis of the vagal effect on the pacemaker and the spread of excitation within the heart.

The main nerve trunks and blood vessels on one side of the neck were dissected out routinely in the course of the operation. The corresponding vagal nerve could easily be put on a bipolar silver electrode and directly stimulated. To avoid interference by the tonic parasympathetic activity and reflex effects caused by activation of afferent fibres, the vagal nerve was blocked centrally to the site of the electrical stimulation by a local injection of a one per cent xylocaine solution into the nerve trunk. Stimuli were delivered by a Grass, model S4 C, stimulator. Rectangular, 5-millisecond pulses were used; the frequency ranged from one impulse every other second to 20 impulses per second. Each stimulation period amounted to 10 seconds for every given frequency, starting with low frequencies under constant control of the electrocardiogram, pulse rate and blood pressure. The stimulation strength used was generally between 7 and 10 volts; this usually sufficed to elicit a maximal heart response at a given frequency of stimulation.

The experimental procedures were generally completed within 15 to 20 minutes, with no side-effects whatsoever.

Results.

1. *Direct electrical stimulation.* The left vagal nerve was electrically stimulated in four patients, and the right in two, after the

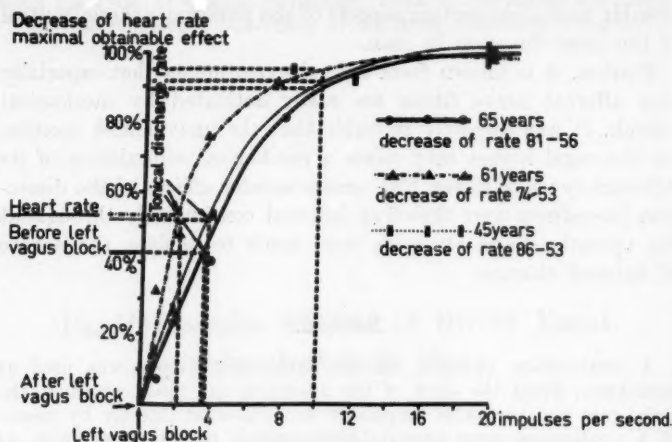


Fig. 1. Effects on the heart rate of direct vagal stimulation with increasing rate.

tonic discharge had been blocked by injection of xylocaine into the nerve trunk, centrally to the site of stimulation. Practically all the fibres to the heart must have been activated by these stimulations, since the heart response did not increase materially when the voltage was further increased at one and the same stimulation rate.

On stimulation of the left vagal nerve, the heart rate was seen to decrease maximally by about 40 per cent, whereas right vagal stimulation induced a considerably more pronounced bradycardia.

Since the available facilities did not permit complete elimination of the stimulation pulses from the ECG record, it was difficult to analyse the ECG in detail during vagal stimulation at higher rates. Some information about the effect on the ECG could nevertheless be obtained by studying the first heart cycle immediately upon interruption of the stimulation, since the vagal effect did not disappear instantaneously.

As shown by fig. 1 the correlation between stimulation frequency and cardiac response forms a hyperbolic curve, where almost maximal effects are reached already at 8 to 10 impulses per second. This was also the case with the change of the P-R interval. It is thus evident that almost the whole range of effector responses can be covered by changes in the discharge rate within

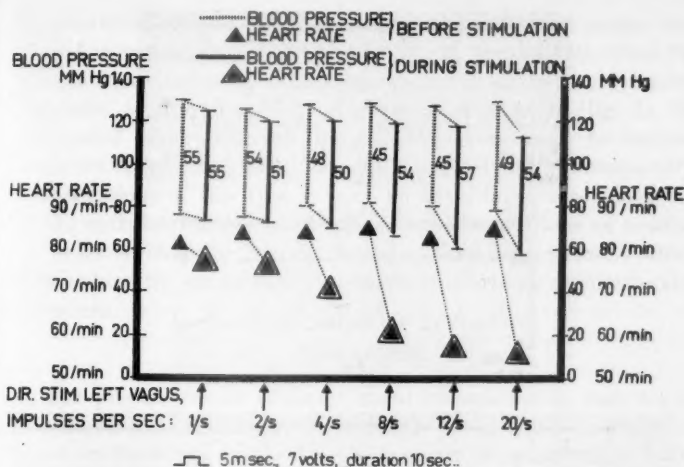


Fig. 2. Arterial blood pressure and heart rate during left vagal stimulation with increasing frequency.

0–10 impulses per second and that even minor shifts in fibre activity must affect the heart considerably.

When — as in the present cases — only one of the vagal nerves was electrically activated, the establishment of ectopic pacemakers was never observed, but this was sometimes seen when the heart was reflexly influenced by activation of afferent vagal fibres (see 2). It should be recalled, however, that the reflex influence on the heart may be more extensive, as it involves concomitant reciprocal changes in the discharge of the two vagal nerves and the sympathetic fibres to the heart.

When a comparison was made between the heart rate before vagal xylocaine block and the effects obtained by the direct stimulations, the tonic control of the heart was closely imitated by a stimulation rate around 2–4 impulses per second (fig. 1). This was also the case when the changes in the P–R interval were compared.

The effects on the arterial blood pressure of a direct stimulation of the vagal heart fibres are illustrated in fig. 2. It is seen that the slowing of the heart was accompanied by a proportional fall in arterial blood pressure, affecting both the systolic and the diastolic pressure, whereas the pulse pressure increased somewhat.

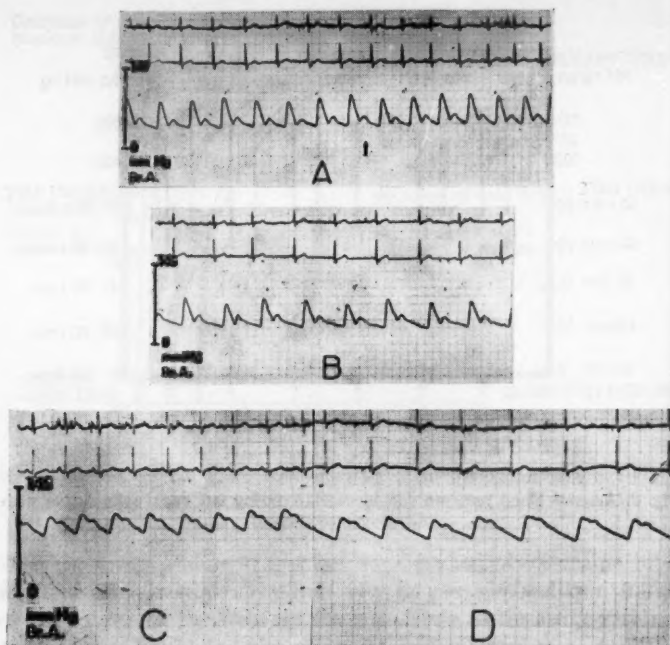


Fig. 3. Effect on the pacemaker of mechanical stimulation of the left vagal nerve during operation before (A and B) and after vagal block (C and D). The arrow in A indicates the end of mechanical, digital stimulation of the nerve. B. another period of mechanical stimulation. C. stimulation peripherally and D. centrally to the xylocaine block.

This was regularly observed, and in no case did the pulse pressure decrease significantly on activation of the vagal heart fibres.

2. *Mechanical stimulation of the vagal nerves during routine operation.* It is apparent that mechanical treatment of the vagal nerves during neck dissection may stimulate fibres that cause changes in the cardiac rhythm and rate. When the ECG and arterial blood pressure were recorded continuously throughout the operation, traction of the vagal nerve was often observed to result in a blood pressure fall, bradycardia and establishment of an ectopic pacemaker. The question then arose whether these effects were induced directly by way of activation of efferent vagal fibres, or if it was a reflex response due to excitation of afferent fibres in the nerve. It soon became evident that these

cardiovascular changes were predominantly of reflex origin, and therefore caused by activation of afferent vagal fibres, since the effects were eliminated if the vagal trunk was blocked by xylocaine centrally to the site of the mechanical stimulation (fig. 3). No activation of the efferent fibres to the heart could be induced even by fairly rough handling of the nerve trunk. Consequently, in operations of this type it may be advantageous to block the nerve with local anesthesia as close to the cranial base as possible in order to stop the impulse traffic in its afferent fibres, which will reduce the occurrence of sometimes alarming cardiovascular reactions.

Discussion.

The present results of direct vagal stimulation in man are in agreement with observations in animals concerning the correlation between discharge rate and heart response (ROSENBLUETH 1950). It is known that most autonomically innervated structures, including sympathetic neuroeffectors in man, show similar characteristics (see FOLKOW and HAMBERGER 1956). The observations concerning the vagal heart control indicate that discharge variations between zero and up to 10 impulses per second will cover practically the whole range of obtainable effector responses. This suggests that vagal tone under normal conditions is maintained by relatively low discharge rates. It was also evident from fig. 1 that the tonic vagal effect on the heart could be closely imitated by direct stimulations of the heart fibres at frequencies around 2—4 impulses per second. Neurophysiological studies of the tonic activity in sympathetic nerves indicate that the majority of the fibres seem to be concomitantly active at a fairly uniform, low rate (see FOLKOW 1955), and sympathetic tone in man seems to be maintained by a discharge rate of about 1—2 impulses per second (FOLKOW and HAMBERGER 1956). Provided that such an activity pattern also holds for the parasympathetic heart fibres, which is *a priori* likely, the 'resting' vagal tone may be evaluated to about 2—4 impulses per second. It is not possible at present, however, to exclude definitely the possibility that only a fraction of the vagal heart fibres are concomitantly active, but then discharge at high rates.

The fact that even on maximal activation of the vagal heart fibres the amplitude of the pulse pressure was not found to decrease, but on the contrary generally increased somewhat, calls



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for some comments. It has been suggested that the prompt and often marked reflex decrease in pulse amplitude, which has been observed on baroreceptor activation in man, might be caused by vagal fibres with a negative inotropic effect on the heart ventricles (PETERSON 1951). In a recent study (CARLSTEN *et al.* 1956, 1957) the sinus nerve in man was directly stimulated at various frequencies and strengths and, here as well, it was regularly noted that a prompt and often marked reduction in pulse pressure formed one of the most striking features of the reflex response. The pulse pressure was not infrequently reduced by some 30–40 per cent, indicating a drastic reduction in stroke volume although no quantitative evaluations are possible. The fact that a direct stimulation of the vagal heart fibres does not reduce the pulse pressure, makes it unlikely that vagal fibres exert a negative inotropic effect on the ventricles of the heart. It then follows that the reflex decrease of stroke volume must be a consequence of a generalized sympathetic inhibition. It has been observed in cats that the effect on the heart of low-frequency sympathetic stimulation is often fairly rapidly eliminated on interruption of stimulation. Consequently, a reflex inhibition of accelerans tone may quite rapidly eliminate the positive inotropic effect of the sympathetic nerve fibres on the heart ventricles (FOLKOW, LÖFVING and MELLANDER 1956). It should further be pointed out that a generalized reflex inhibition of sympathetic tone affects also the venous side of the cardiovascular system (ALEXANDER 1954, PAGE, HICKAM, SIEKER, McINTOSH and PRYOR 1955). This must result in some widening of these reservoir vessels, where even a slight pooling of blood must cause a definite reduction in venous return to the heart ventricles. These consequences of a sudden reflex inhibition of sympathetic tone probably explain the prompt reduction in stroke volume in man when baroreceptor fibres are activated.

The present investigation indicates that afferent fibres in the vagal nerve are often stimulated mechanically during routine neck dissection, and that this stimulation sometimes can have profound reflex effects on the cardiovascular system. From this observation the practical conclusion can be drawn that a local xylocaine block of the vagal nerve, as cranially as possible in the neck, should be a good measure to prevent some of the cardiovascular complications that may occur in surgical operations of this type.

Summary.

The vagal control of the heart has been studied in man during total neck dissections. After that the normal tonic discharge of the fibres to the heart had been blocked by locally injected xylocaine, the nerve could be directly stimulated at maximal strength and increasing frequencies. Plotting the effector response, as measured by the pulse rate, ECG and blood pressure, against the stimulation frequencies gives a hyperbolic curve, typical of autonomic neuroeffectors, with almost maximal effects on the heart already at 10 impulses per second.

By comparing the heart response to known stimulation rates with the heart rate before nerve block, the normally present vagal tone could be roughly evaluated to about 2—4 impulses per second.

No evidence was obtained in support of vagal fibres with a negative inotropic effect on the heart ventricles, as has previously been suggested.

It was further observed that afferent fibres in the vagal nerves with reflex connections to the vasomotor centre are very sensitive to mechanical stimuli so that dissections of the nerve can induce drastic reflex effects on the cardiovascular system.

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Ventilatory Studies of the Lungs During Continuous Pressure Breathing.

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In his review "Intrapulmonary Distribution of Inspired Gas", FOWLER (1952) expresses his opinion thus: "... In an adult subject sitting at rest, the normal lung is not uniformly ventilated, but some alveolar spaces have a more rapid turnover rate or "deeper" ventilation than others ...". This view appears to be shared by most authors. Extensive references to the literature have been made by RAUWERDA (1946) and FOWLER (1952) in their reviews on the subject. Continuous recording of the elimination of nitrogen from the lungs during oxygen breathing has proved most adaptable in elucidating these conditions, and LUNDIN (1955) has shown that, in normal subjects, it is adequate to follow end-tidal air nitrogen percentage breath by breath during oxygen breathing to attain a quantitative measure of the lung ventilation. The degree of uneven ventilation is higher in elderly subjects (ROBERTSON, SIRI and JONES 1950, COMROE and FOWLER 1951, FOWLER, CORNISH and KETY 1952, GEORG 1955) and in different lung diseases (DARLING, COUNAND and RICHARDS 1944, FOWLER 1949, BRISCOE, BECKLAKE and ROSE 1951, FOWLER et al. 1952, GEORG 1955) and is, therefore, generally recognized as a measure for the ventilatory efficiency of the lungs.

The effect of variations in end-expiratory lung volume on the evenness of alveolar ventilation has not been established. RAUWERDA (1946) observed a higher degree of even ventilation when the ratio $\frac{\text{depth of inspiration}}{\text{lung volume}}$ increased, whilst FOWLER (1954)

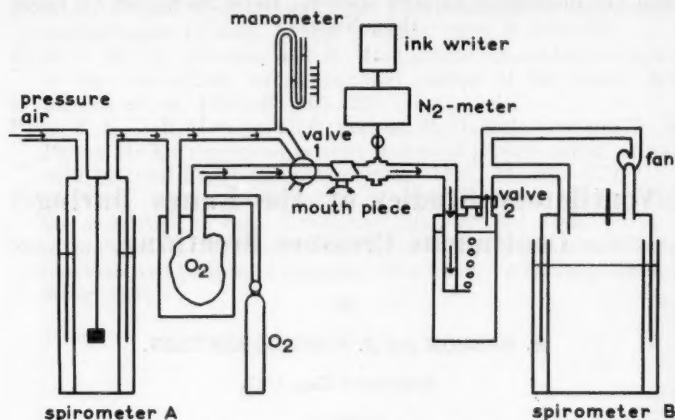


Fig. 1. Elementary diagram of the apparatus.

found that the degree of uneven ventilation remained unchanged in experiments with constant tidal volume at different levels of pulmonary inflation. BOUHUYS, JÖNSSON and LUNDIN (1957) found no significant differences during moderate degrees of exercise where the tidal volume ranged from about 500 to 2,500 ml.

The purpose of this investigation is to elucidate the degree of uneven ventilation by continuous pressure breathing (CPB) as compared with normal breathing. As pressure breathing frequently leads to hyperventilation and increased tidal volumes, we have also investigated the degree of uneven ventilation with varying tidal volumes. At the same time, other measures for ventilatory efficiency of the lungs have been investigated: nitrogen washout time (COLLDAHL and LUNDIN 1952), nitrogen washout volume (GEORG 1955) and lung clearance index (BECKLAKE 1952).

Methods and Procedure.

Fundamentally, the apparatus is the same open-circuit system as that used by other authors (ROBERTSON et al. 1950, FOWLER et al. 1952, LUNDIN 1955, GEORG 1955) during similar investigations under normal pressure but with the requisite modifications for continuous pressure breathing (Fig. 1). In the spirometer A, a weight is placed on the bell so that the air in the spirometer is under positive pressure. Tubes run from the spirometer down into a sealed glass container and thence to valve 1 located directly in front of the mouthpiece. A manometer (U-pipe) is inserted in this tube for recording pressure on the

inspiratory side. The air which is under positive pressure in the spirometer A can be replenished as required. Oxygen can be filled in a rubber bag located inside the sealed glass container with connecting tube to valve 1. Thus the oxygen will remain under the same positive pressure as the air. Valve 1 can either supply air under atmospheric pressure or air and oxygen, respectively, under positive pressure. Under normal pressure, the oxygen is filled into a bag connected to valve 1 which, in turn, supplies either air or oxygen. Expiratory air passes a needle valve, through which air is continuously extracted for analysis in the nitrogen meter. The bulk of the expiratory air continues into a sealed container where a metal tube leads off under water. The tube can be moved upwards and downwards. The height under water determines the pressure on the expiratory side which is adjusted to 1–2 cm water more than the pressure on the inspiratory side. A flutter valve is located at the lower end of the tube to avoid reflux of the water during inspiration. The gas effervesces through the water and, from now on, provides atmospheric pressure. Valve 2 is located at the mouth of the container and permits the escape of gas into the air or into the spirometer B. The container is dispensed with under normal pressure — in which case, valve 2 is placed directly distal of the needle valve. By means of a fan, which circulates 80 litres gas per minute in the spirometer system, the gas can be mixed and samples can be extracted for analysis.

During pressure breathing the pressure has oscillated from 17–20 cm water during inspiration to 20–21 cm water during expiration.

We have used a nitrogen meter, Lundin—Åkesson model. Before the gas enters the nitrogen meter for analysis, the moisture is arrested by cooling (carbon dioxide ice plus alcohol).

The nitrogen meter is calibrated against gas analyses (Scholander half-cc gas analyzer). A calibration curve has been constructed where N_2 percentage is plotted against the mm readings on the ink writer. The calibration of the scale 0–100 is checked frequently. Deviation of the gas analyses from the calibration curve was found to be from +0.8 to –0.8 per cent for the first 48 specimens. Standard deviation of the difference 0.4 per cent. This then comprises the total of erroneous readings on the ink writer and faulty analysis in the nitrogen meter. The sensitive scale (0–10) is calibrated for each day of experiment.

Accurate instructions are given to the subject before commencing. He rests for some minutes and then sits comfortably in a chair while being connected to the apparatus. He firstly breathes air under atmospheric pressure and then air under positive pressure until respiration becomes regular and with maximum constant tidal volume. During this phase, valve 2 is placed so that the expiratory air can escape into the air after having effervesced through the water in the container. At the end of an expiration, valve 1 is adjusted so that the subject receives oxygen under positive pressure. During this primary inhalation of oxygen, valve 2 is adjusted so that the expiratory air enters the spirometer B. The nitrogen concentration in the expiratory air can be observed on the ink writer, and the experiment ceases only after the N_2 concentration has remained very low for two minutes, thus enabling

all the N_2 in the tubes and in the gas above the level of the water in the container to go over to spirometer *B*. Valve 2 is then switched over during an inspiration and the experiment comes to an end.

The volume of spirometer *B* and tubes from valve 2 is about 15 litres at commencement. The gas has a nitrogen concentration of 6–10 per cent. The volume of the spirometer is read at each expiration. Before commencement and at the end of the experiment, the gas is mixed and analyzed.

End-tidal air nitrogen percentage is then read from the curve for every individual respiration. Corrections are made for nitrogen in the oxygen (0.3 %) and for nitrogen eliminated from blood and tissues. The following values are applied for N_2 elimination during oxygen breathing (LUNDIN 1955): 1 min. 50 ml, 2 min. 50 ml, 3 min. 30 ml, 4 min. 25 ml and 5 min. 20 ml. The correction in per cent is thus tissues' N_2 in ml per minute $\times 100$

$$\text{resp. freq.} \times (V_T - V_D)$$

where V_T = tidal volume, V_D = dead space volume and resp. freq. = frequency of respiration.

V_D , during normal and stable respiration, is set at 150 ml. With increasing V_T , V_D becomes greater and constitutes about 25 per cent of V_T . Maximum inspiration increases V_D by about 100 ml. (FOWLER 1948).

Accordingly, a certain V_D is assumed for a given V_T : V_T less than 600 ml — V_D = 150 ml. V_T 600–900 ml — V_D = 150–220 ml (25 per cent of V_T). V_T 900–1,250 ml — V_D = 230 ml. V_T 1,250–2,000 ml — V_D 240 ml. V_T greater than 2,500 ml — V_D = 250 ml.

V_D increases during CPB, but we have not found any accurate data on this, and the same values have been used as for normal pressure.

An error in the correction due to a miscalculation in the presumed V_D is, however, of little importance as compared with other experimental errors. Corrected end-tidal air N_2 % =

$$N_2 \% \text{ measured} - 0.3 - \frac{\text{tissues' } N_2 \text{ in ml per min.}}{\text{resp. freq.} \times (V_T - V_D)}$$

On the basis of the corrections eliminating the influence of nitrogen in the oxygen and the elimination from blood and tissue, the following equation will, with even ventilation, apply:

$$F_{A_n} = F_{A_0} \times W^n \quad (1) \text{ where}$$

F_{A_0} means the fraction of N_2 in the alveolar air at commencement

F_{A_n} means the fraction of N_2 in the alveolar air after n respirations.

W = "alveolar dilution factor" and is $\frac{\text{expir. } V_L}{\text{expir. } V_L + V_T - V_D}$

The term expiratory lung volume (expir. V_L) means, in this essay, the lung volume at completed expiration — at normal expiratory level equivalent to *FRC*, at maximum expiration to *RV*.

An exponential function as in equation (1) gives a straight line in a semi-logarithmic system, where $\log N_2$ % is plotted against the number of respirations. A straight line will, therefore, show even ventilation.

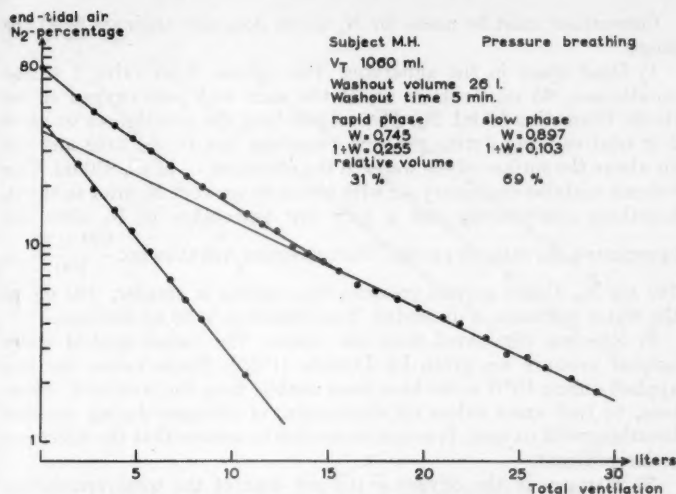


Fig. 2. Graphic analysis of experiment with uneven ventilation, showing the two fractions and their volume in per cent of expiratory lung volume.

Deviations from this line will show uneven ventilation. The straight line will also appear when $\log N_2 \%$ is plotted against ventilated volume (GEORG 1955). We have chosen this as it simplifies the calculations — particularly with greater V_T .

When deviations from the straight line are observed, it is possible, through mathematical analysis, to divide the lung into two or more fractions — each fraction with even ventilation. The analysis follows the method used by LUNDIN (1955). Similar analysis is described by FOWLER (1952).

For each experiment, the number of fractions and their volume is calculated in per cent of expiratory lung volume (Fig. 2). All volumes are given as *BTPS* values. For pressure breathing, the volumes are given at body temperature, barometric pressure + 15 mm Hg, saturated "*BT(P + 15)S*".

Nitrogen washout volume is read from the curve — such volume being the total ventilation required to reduce end-tidal air nitrogen percentage to 2.5 %.

Nitrogen washout time means the time required to reach 2.5 per cent, thus:

$$\frac{\text{nitrogen washout volume}}{\text{ventilated volume in litres/min.}}$$

Expiratory lung volume is determined in accordance with COURNAND, BALDWIN, DARLING and RICHARD's open circuit method (1941) where

$$FRC = \frac{\text{ml } N_2 \text{ collected during the experiment}}{N_2 \% \text{ in lungs at start} - N_2 \% \text{ in lungs at finish}}$$

Corrections must be made for N_2 which does not originate from the lungs:

1) Dead space in the apparatus: The volume from valve 1 to the mouthpiece, 65 ml, contains air at the start and pure oxygen at the finish. Correction 50 ml N_2 . The volume from the mouthpiece to valve 2 is relatively big during pressure breathing due to the tube and the air above the surface of the water in the container — in all, 950 ml. This volume contains expiratory air with about 80 per cent N_2 prior to the O_2 breathing commencing and a very low percentage of N_2 after the experiment has come to an end. The correction will thus be: $\frac{950 \times 80}{100} =$

760 ml N_2 . Under normal pressure, this volume is smaller, 100 ml, as the water container is discarded. The correction is 80 ml nitrogen.

2) Nitrogen eliminated from the tissues: The values applied under normal pressure are given by LUNDIN (1955). These values are also applied during CPB as we have been unable, from the available literature, to find exact values for elimination of nitrogen during pressure breathing with oxygen. It seems reasonable to assume that the difference is insignificant.

3) Nitrogen in the oxygen = 0.3 per cent of the total ventilation. N_2 % in the lungs at start (about 81 % — Cournand et al. 1941) — N_2 % at finish is set at 80 %.

Checking the findings of expir. V_L under normal pressure was carried out by means of a closed circuit technique (VOGT LORENTZEN 1953). A bag with known volume was then ventilated with the experimental set-up as for CPB and the volume was determined by the said open circuit method. Under both conditions, satisfactory accuracy was achieved.

BECKLAKE (1952) has declared her "lung clearance index" as litres ventilation required to wash 90 % F. R. A. free of N_2

90 % F. R. A.

This index may be considered as nitrogen washout volume per litre of expir. V_L and we have chosen to quote it as $\frac{\text{nitrogen washout volume}}{\text{expir. } V_L}$ as this is more apparent from the curves.

Results.

Experiments have been carried out on an artificial lung similar to that constructed by BECKLAKE (1951).

The artificial lung with even ventilation: Fig. 3 shows four experiments, all of which gave a straight line when graphically analyzed. The artificial lung with uneven ventilation: It has been possible to separate the two phases — even with a rapid phase of only 10 per cent of the lung volume. During six experiments on the same model, it was found that the slow phase comprised

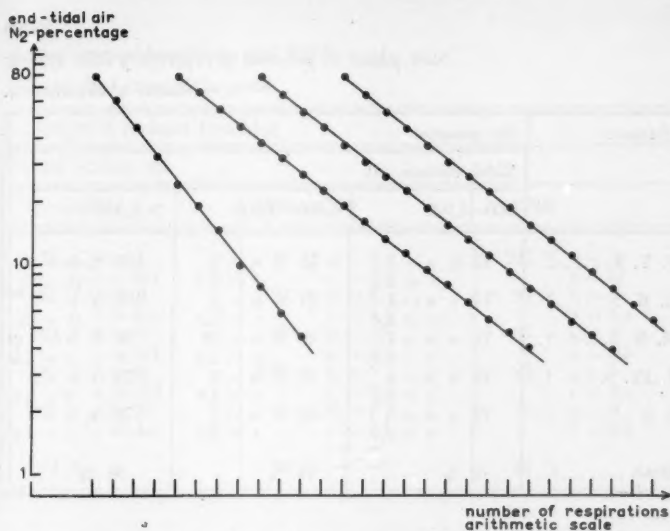


Fig. 3. Graphic analysis of 4 experiments with evenly ventilated artificial lung.

33, 31, 33, 31, 31 and 30 per cent, respectively, of the lung volume.

Test subjects are male members of the laboratory staff without trace of lung disease — one 48 years old, the others in the age group of 20—28 years. Altogether, 68 experiments were made on the latter five subjects at normal pressure — hereof 27 with determination of expir. V_L . 67 experiments were carried out with CPB — 27 with determination of expir. V_L .

From all experiments at normal pressure and with tidal volumes 500—1,000 ml, analysis has shown that the lungs behave as if there were two fractions with different rates of nitrogen elimination. The slow phase comprises, on an average 72 per cent of the lung volume (range 58—88 per cent). Variations from one subject to another are insignificant.

Table 1 illustrates conditions with different V_T . On subjects F. V. L. and O. K. an increasing degree of even ventilation is shown with greater V_T , and altogether five experiments have shown even ventilation with large V_T . On subject M. H. there is possibly a slightly increased even ventilation, and on the two others no change with increasing V_T .

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Table
Slow phase in per cent of expiratory lung volume

n = number of experiments.

Subject	No pressure		
	Tidal volume, ml		
	500—1,000	1,000—2,500	> 2,500
F. V. L.	72 % n = 3 ε = 1.4	75 % n = 3 ε = 2.9	100 % n = 3 (ε = 0)
O. K.	73 % n = 4 ε = 4.6	87 % n = 4 ε = 5.9	100 % n = 2 (ε = 0)
M. H.	71 % n = 7 ε = 3.3	71 % n = 12 ε = 1.8	88 % n = 6 ε = 3.2
J. Ph. S.	75 % n = 4 ε = 5.5	76 % n = 3 ε = 1.0	73 % n = 5 ε = 4.9
I. S.	72 % n = 5 ε = 2.7	68 % n = 2 ε = 3.0	78 % n = 3 ε = 7.5
Mean	72 %	74 %	86 %

CPB shows similar results. In all experiments with V_T 500—1,000 ml, the analysis has shown two fractions — the slow fraction averaging 79 per cent of the lung volume, range 60—90 per cent. As will be seen from Table 1, increasing V_T gives a higher degree of even ventilation on subjects F. V. L. and O. K. Altogether, five experiments showed even ventilation with large V_T . On the remaining three subjects, no change was observed.

Fig. 4 illustrates nitrogen washout volume with varying V_T . A great change takes place from normal respiration to *CPB*. The washout volume with *CPB* is considerable with the smallest V_T and the volume decreases as V_T increases. At normal pressure, the washout volume decreases only slightly with increasing V_T .

Fig. 5 shows that the nitrogen washout time decreases with increasing alveolar ventilation. With constant alveolar ventilation, the washout time is somewhat longer during *CPB* than during normal respiration. This finding was made on all subjects.

Table 2 illustrates the lung clearance index which does not change conclusively from normal respiration to *CPB*, nor with varying V_T .

1.

during normal breathing and CPB.

 \pm = standard error of the mean.

Continuous pressure breathing		
Tidal volume, ml		
500—1,000	1,000—2,500	> 2,500
78 % n = 5 \pm = 2.1	87 % n = 2 \pm = 3.5	100 % n = 3 (\pm = 0)
81 % n = 4 \pm = 0.7	76 % n = 3 \pm = 1.7	98 % n = 3 \pm = 1.7
80 % n = 8 \pm = 1.9	80 % n = 12 \pm = 3.3	85 % n = 4 \pm = 5.9
82 % n = 3 \pm = 1.9	82 % n = 5 \pm = 3.7	86 % n = 3 \pm = 6.5
68 % n = 3 \pm = 4.0	66 % n = 4 \pm = 0.7	73 % n = 3 \pm = 3.2
79 %	78 %	88 %

Discussion.

Experiments with the artificial lung show that the method is sufficiently accurate and should be well-adapted for investigating the nitrogen clearance of the lungs under varying conditions. In experiments conducted on human subjects, we found that the degree of uneven ventilation and the variations on one individual were the same as those found by previous authors (FOWLER et al. 1952, LUNDIN 1955). With increasing V_T , an increasing degree of even ventilation is found on some subjects, while on others there is no change. The slow phase constitutes a somewhat greater percentage of lung volume with V_T 500—1,000 ml. during CPB than at normal pressure for the first four subjects. No change occurred on subject I. S. The change is too slight to assert that CPB implies more even ventilation. These findings are not contributory factors to the discussion on the causes of uneven ventilation.

COLLDAHL and LUNDIN (1952) used nitrogen washout time as a measure for the ventilatory function on asthmatic patients. COURNAND et al. (1941) used alveolar N_2 concentration after 7 minutes of oxygen breathing (pulmonary emptying rate) — this is fundamentally the same. But, as the authors themselves admit, nitrogen washout time is greatly dependent on the alveolar

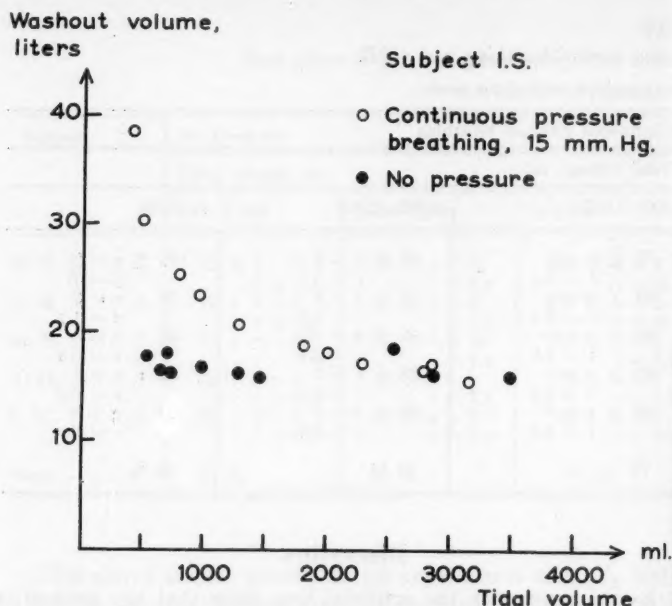


Fig. 4. Nitrogen washout volume in relation to tidal volume during normal breathing and *CPB*.

ventilation which can be voluntarily influenced (Fig. 5). GEORG (1955) points out that nitrogen washout volume gives more valuable information. It appears from Fig. 4 that nitrogen washout volume during *CPB* is very large with small V_T and decreases sharply when V_T increases, whilst under normal pressure it decreases much less. This finding was made on all subjects with the exception of F. V. L. An attempt has been made to explain this finding by determining the lung clearance index. It has been found (Table 2) that the lung clearance index is virtually independent of V_T , and the same with normal breathing as with *CPB*. The large washout volume at small V_T during *CPB* can thus be explained by the fact that the lungs are then expanded and have a very large volume. When V_T changes, variations in expir. V_L are much smaller during normal breathing.

With subject F. V. L., the conditions were somewhat different. Five experiments were made during *CPB* with V_T 500–800 ml. In one of these experiments, the washout volume was 29.0 l. —

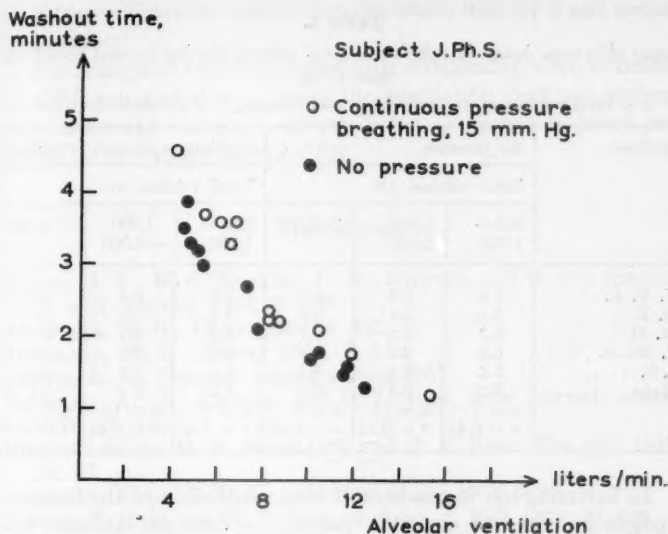


Fig. 5. Nitrogen washout time in relation to alveolar ventilation during normal breathing and CPB.

in the other four 17.8—20.4 l. These results can reasonably be explained in that the subject in one experiment was relaxed and breathed with large expir. V_L . In the four other experiments, he had smaller expir. V_L in spite of the small V_T . The washout volume thus remained small.

As subject F. V. L. is the eldest (48 years), it could be assumed that the difference in age had a bearing on the findings. However, similar results were experienced with two younger subjects under normal pressure (O. K. 20 years old and M. H. 28 years old).

(O. K.: V_T 684 ml, expir. V_L 4.7 l, lung clearance index 5.4 and washout volume 25.4 l, as against 19.3 l at corresponding V_T and normal expiratory level.)

M. H.: V_T 639 ml, expir. V_L 4.3 l, lung clearance index 5.4 and washout volume 23.6 l, as against 18.8 l at corresponding V_T and normal expiratory level.)

Breathing with small V_T in the inspiratory reserve volume under normal pressure thus gives increased washout volume with constant lung clearance index — identical with conditions during CPB where a small V_T usually involves a large expir. V_L . The age, therefore, does not seem to be of importance in this connection.

Table 2.

Lung clearance index in relation to tidal volume during normal breathing and CPB.

n = total number of experiments in each column, σ = standard deviation.

Subject	No pressure			Continuous pressure breathing		
	Tidal volume, ml			Tidal volume, ml		
	500— 1,000	1,000— 2,500	> 2,500	500— 1,000	1,000— 2,500	> 2,500
F. V. L.	5.2	5.2	6.2	5.8	5.3	5.3
O. K.	5.4	5.5	5.6	6.4	5.3	5.0
M. H.	5.2	5.6	6.1	6.3	5.4	5.7
J. Ph. S.	6.3	6.0	6.6	5.9	5.3	5.5
I. S.	5.4	5.2	6.8	5.8	5.2	5.4
Mean	5.5	5.5	6.3	6.0	5.3	5.4
	$n = 7$ $\sigma = 0.44$	$n = 11$ $\sigma = 0.53$	$n = 9$ $\sigma = 0.83$	$n = 9$ $\sigma = 0.56$	$n = 10$ $\sigma = 0.17$	$n = 8$ $\sigma = 0.38$

In summing up, it can be said that ventilation of the lungs — judged by nitrogen clearance pattern — does not undergo any change when switching from normal breathing to CPB.

When BITTER (1957) finds that "... the ventilation-perfusion relationship appears to be markedly altered with pressure breathing as seen by the increased ($P_a - P_A$) CO_2 gradient ...", whilst our findings show unchanged ventilation, this can probably be attributed to a changed perfusion when switching to CPB. It is generally recognized that blood perfusion of the lungs changes under CPB. FENN, OTIS, RAHN, CHADWICK and HEGNAUER (1947) found that an increase of pulmonary pressure of 30 cm of water displaces 500 cc, or about half of the blood contained in the lungs.

Summary.

The nitrogen clearance of the lungs during oxygen breathing has been investigated by means of the nitrogen meter during continuous pressure breathing, compared with normal conditions.

In all, 135 experiments have been made on 5 healthy subjects. The degree of uneven ventilation, nitrogen washout time, nitrogen washout volume and lung clearance index have been investigated.

Increasing tidal volumes gave more even ventilation on some subjects but no change on others.

Nitrogen clearance pattern remains stable with *CPB* and normal respiration.

The changes in ventilation-perfusion relationship when switching to *CPB* can probably — when the ventilation does not undergo any important change — be attributed to change in blood perfusion of the lungs during *CPB*.

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Cold Defense Reactions Elicited by Electrical Stimulation within the Septal Area of the Brain in Goats.

By

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The common view held about central regulation of body temperature is that there exists a "centre" controlling heat loss mechanisms in the preoptic area and adjacent parts of the anterior hypothalamus and that the central regulation of heat conservation mechanisms is exerted from the posterior hypothalamus (BAZETT 1949). The localization of the "heat loss centre" to the preoptic area and the anterior hypothalamus has been demonstrated in a number of investigations (MAGOUN, HARRISON, BROBECK and RANSON 1938, ELIASSON and STRÖM 1950, v EULER 1950, ANDERSSON, GRANT and LARSSON 1956, and others). Clear evidence for the existence of a more localized "centre" regulating the heat production mechanisms have however not been given. Earlier experiments in which lesions were placed in various parts of the brain stem do indicate that the two mechanisms can be separated from each other and that the descending fibres from the structural elements subserving the mechanisms of heat production run more medially in the mesencephalon than some of the fibres concerned with the mobilization of heat loss mechanisms (KELLER 1938). BIRZIS and HEMINGWAY have recently (1956) determined the brain stem pathway for shivering in the cat and the same authors have later (1957 a) been able to produce shivering by electrical stimulation, at different sites along this pathway. The most anterior points where these stimulations caused shivering were found to be medially in the tuberal region

of the hypothalamus. Shivering as a result of brain stimulation seems otherwise only to have been reported by AKERT and KESSELING (1951). These authors made an analysis of the experiments in which HESS had observed shivering as result of electrical stimulation in the unanaesthetized cat and they found that the sites of stimulation in 10 out of 11 cases had been in, or in the close vicinity of the septum pellucidum. They also made the interesting observation that it often had been difficult to repeat a positive result at a given point.

In a series of experiments performed to study the effect of prolonged stimulation of the preoptic "heat loss centre" in the unanaesthetized goat (ANDERSSON and PERSSON, in preparation) it was observed that stimulations medial and dorsal to this "centre" produced shivering. The result of a further investigation of this phenomenon is presented in this paper.

Methods.

Three adult female goats were used for the experiments. HESS' technique (1932, 1949) was used for the electrical stimulations making it possible to stimulate at three different points in the brain at each horizontal level. The use of nichrome electrodes made it possible to leave the electrodes in position in the brain for several days so that one and the same animal could be used for more than one experiment. The parameters of stimulation were 1.5 to 3.5 V and 50 cycles per sec.

When needed, the rectal temperature was continuously recorded by help of a thermocouple connected to an "Ellab" thermometer and in the same way the skin temperature of the ears or the nose could be recorded with a thermocouple attached to the shaved skin.

For a study of the effect of electrical stimulations in high environmental temperature the goats were placed in an electrically heated chamber, where the temperature was kept constant at a desired level by means of a thermostat. A low environmental temperature, on the other hand, was obtained by placing the goats in a cool room at about 4° C, and a rapid fall of the body temperature of the goats was produced by giving cold water by stomach tube into the rumen.

Electromyograms from the shivering muscles were obtained using an ECG-apparatus connected to needles fixed subcutaneously over an active muscle.

Histological methods. After the animals had been killed their heads were perfused first with Ringer's solution and then with Bodian's fluid and the brains were fixed in the latter and embedded in celloidin. Serial transverse sections, 100 microns thick, were made through that part of the brain which had been the site of the electrodes and the sections were as much as possible directed parallel to the electrode tracks. The sections were stained with toluidin blue.

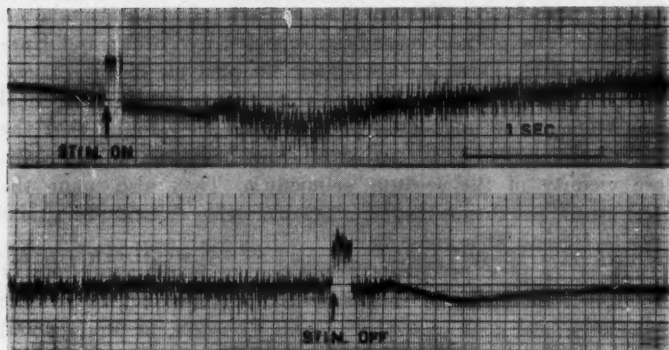


Fig. 1. An electromyogram of shivering muscles taken during a short period of electrical stimulation in the septal area of a goat kept in room temperature.

Results.

When studying the effect of electrical stimulation of the pre-optic "heat loss centre" in the goat (ANDERSSON, GRANT and LARSSON 1956) the electrodes were generally placed somewhat lateral to the midline. When, however, in the experiments reported here the electrodes were purposely placed at approximately the same transverse plane but along the midline of the skull, it was found that stimulations at sites somewhat above and medial to the "heat loss centre" had the opposite effect to an activation of this "centre". Thus shivering, peripheral vasoconstriction, slowing of respiration, a moderate rise in rectal temperature and sometimes piloerection and a huddling up of the animals were obtained as effects of stimulation. A more detailed study of some of these phenomena revealed the following.

Stimulations in room temperature (20° C).

Electromyograms were taken before, during and after shorter periods of effective stimulation to study the character of shivering. The latency before its onset was 0.5 to 1 sec. and the response stopped within less than a second after the stimulus was switched off (Fig. 1). However, if the period of stimulation was prolonged for several minutes the intensity of shivering gradually diminished and after a varying period of time the shivering stopped completely. This usually happened when the rectal temperature had

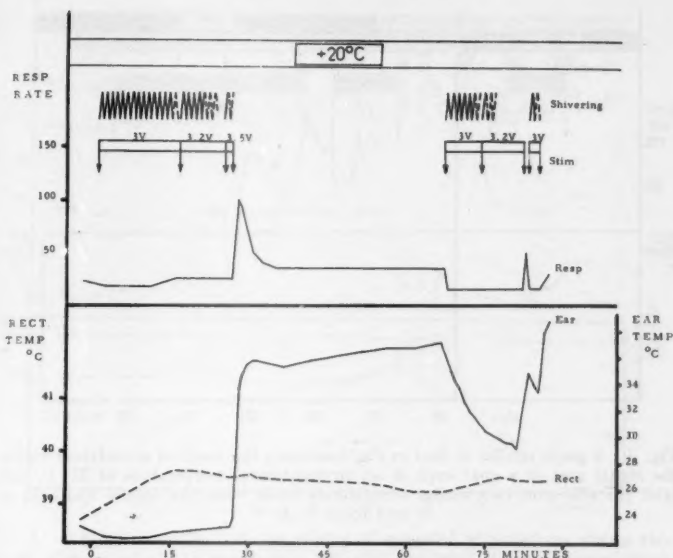


Fig. 2. A graph showing the shivering response and the effects on respiration rate, rectal temperature and ear surface temperature obtained by stimulation within the septal area of a goat kept in room temperature.

risen 0.2 to 0.5°C . An increase of stimulus strength at this stage could temporarily cause the reappearance of shivering. The respiratory frequency — in most cases being low when the stimulations started — stayed down during the periods of stimulation, but if these were prolonged sufficiently to cause a rise in rectal temperature, the respiratory frequency rose markedly just after the end of stimulation and stayed high for a minute or longer. Similarly, if the ear and nose surface temperature was high before the onset of stimulation it started to decline after 1 to $1\frac{1}{2}$ min. of stimulation and stayed down till the stimulation ended, when it rose again. After longer periods of stimulation this rise occurred even if the ear and nose surface temperature had been low when the stimulation started. Fig. 2 illustrates the effects of repeated stimulations in room temperature at a site in the septum pellucidum where shivering and the other above mentioned effects were seen as result of stimulation.

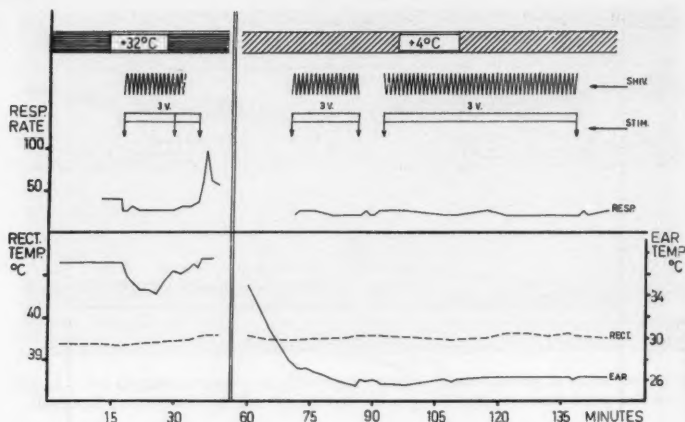


Fig. 3. A graph similar to that in Fig. 2 showing the result of stimulation within the septal area in a goat kept in an environmental temperature of 32° C, and later the effects of two similar stimulations made when the animal was kept in a cool room at 4° C.

Stimulations in an environmental temperature of 4° C and after lowering of the body temperature of the animals.

The gradual disappearance of the shivering effect observed during stimulations in room temperature concomitant with a rise in rectal temperature made it of interest to find out whether this phenomenon also appeared during stimulations in a colder environment. It was found that providing the animal was kept in an environmental temperature of 4° C, the shivering effect persisted throughout very long periods of stimulation (up to 40 min.), and that if any rise in rectal temperature was observed during stimulations in this environment it was small. Fig. 3 shows the course of an experiment in which a goat was first stimulated in an environmental temperature of 32° C and later was taken down for further stimulations in a cool room where the temperature was kept at 4° C.

When the body temperature of an animal kept in room temperature was lowered through the administration of 2 to 3 liters of cold water by stomach tube the rectal temperature fall 1° to 2° C and the goat started to shiver vigorously. Concomitant with the subsequent gradual rise in rectal temperature the intensity of shivering diminished. Stimulations made soon after the

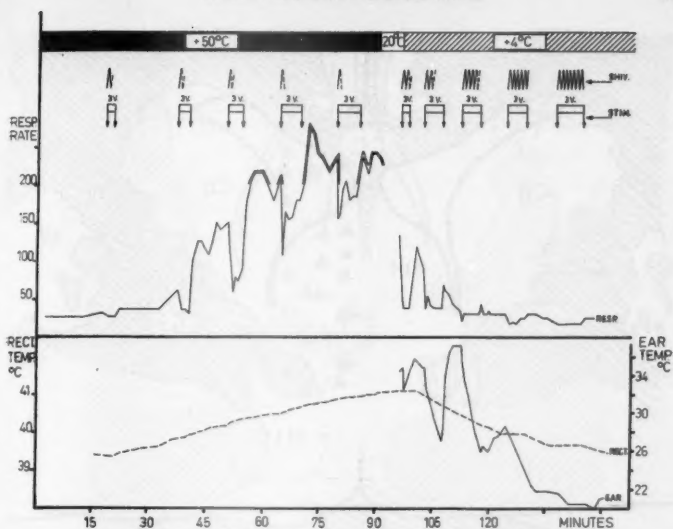


Fig. 4. A graph showing first the effects of repeated stimulations within the septal area in a goat exposed to heat, and later the result of similar stimulations when the animal was taken over into an environmental temperature of 4° C.

Doubled lining of respiration curve indicates polypneic panting.

shivering (induced by the administration of cold water) had stopped completely revealed that at this point in the experiment the shivering effect of stimulation was markedly facilitated, as indicated by the fact that the stimulus strength needed to produce shivering was now considerably lower than it had been before the cold water was given.

Stimulations in an environmental temperature of 50° C.

Stimulations at sites where shivering was produced when the goats were kept in room temperature were also made with the animals placed in a constant temperature room at 50° C. As the environmental temperature now was higher than the body temperature of the goats the ear surface temperature was no longer recorded. The stimulations were started when the animal had been in the constant temperature room for 15 min. to half an hour causing some rise in rectal temperature and of respiratory frequency. Under these conditions the stimulations only caused slight shivering of very short duration. There was a slight tremor

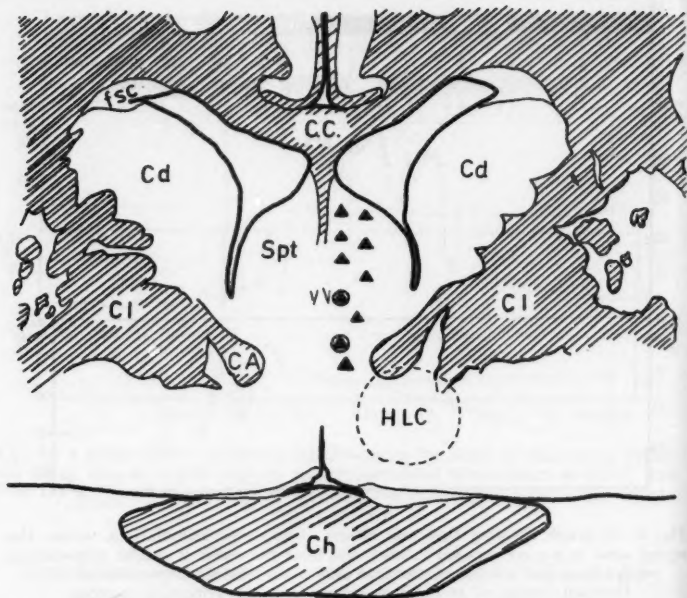


Fig. 5 a. A diagram of a transverse section through the forebrain of a goat slightly in front of the anterior commissure.

Black triangles: Points where shivering, peripheral vasoconstriction and inhibition of polypneic panting were obtained as effects of electrical stimulation.

Encircled triangles: Points where in addition to the above mentioned effects piloerection and a huddling up of the animals were observed.

CA:	commissura anterior
Cc:	corpus callosum
Cd:	N. caudatus
Ch:	chiasma opticum
CI:	capsula interna
fsc:	fasciculus sub-callosus
Spt:	Area septalis.

The encircled area labelled HLC marks approximately the region where electrical stimulation causes the mobilization of heat loss mechanisms in the goat.

just after the onset of stimulation but it vanished within 1 to 2 minutes. The respiratory rate, on the other hand, decreased markedly due to the stimulations. This decrease was most pronounced during the first minute of stimulation. The rise in rectal temperature recorded when repeated stimulations were made in the warm environment was steeper than that observed in control animals kept for the same period of time in this environment.

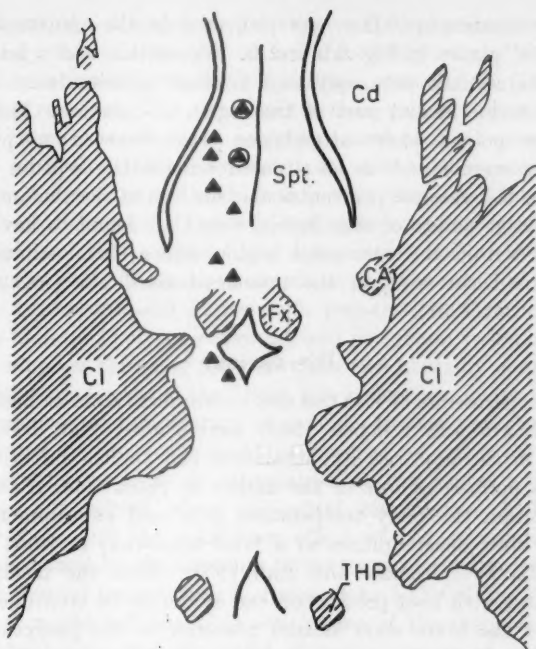


Fig. 5 b. A diagram of a horizontal section through the septal area and the thalamus of the goat at a level slightly dorsal to the anterior commissure.

For symbols see Fig. 5 a.

CA:	commissura anterior
Cd:	N. caudatus
CI:	capsula interna
Fx:	fornix
Spt:	Area septalis
THP:	tractus habenulo-peduncularis.

When an animal was taken from the hot room and shortly afterwards was placed in the cool room (4°C) the shivering effect of stimulation gradually reappeared and a fall of ear surface temperature was observed during the periods of stimulation. Fig. 4 shows the course of an experiment of this kind.

Localization of effective points of stimulation.

The points of stimulation at which the heat conservation reactions were obtained were all situated in or in the close vicinity to the septum pellucidum between the corpus callosum and the

anterior commissure. They are projected in the transverse and horizontal planes in Fig. 5 a and b. Piloerection and a huddling up of the animals were only seen as result of stimulation in the most anterior medial part of the region in question. The most posterior points where stimulation was observed to produce shivering were found to be situated outside the septum in the region of the anterior periventricular nucleus of the thalamus. All the effective points of stimulation were thus found to have been situated within the very same region where HESS' stimulations had caused shivering in the unanaesthetized cat (AKERT and KESSELRING 1951).

Discussion.

The experiments of KELLER and co-workers (KELLER and HARE 1931, BLAIR and KELLER 1946) have shown that destructive lesions in the posterior hypothalamus can abolish shivering and cause a permanent loss of the ability to resist cold. A capacity to maintain the body temperature in a cold environment may persist after decerebration at a level somewhat in front of the pons (BAZETT, ALPERS and ERB 1933). Thus the mechanisms concerned with heat production can evidently be controlled from areas of the brain stem located posterior to the preoptic "heat loss centre". However, the fact that electrical stimulations in the septal area can produce shivering (AKERT and KESSELRING 1951) and, as has been demonstrated in the experiments reported here, simultaneous to the shivering can mobilize other mechanisms concerned with the defense against cold, shows that structural elements exerting a stimulatory action on heat conservation mechanisms are to be found anterior to the hypothalamus. Whether the septal area is the origin of the shivering pathway analysed by BIRZIS and HEMINGWAY (1956, 1957 a and b) or is a part of it remains to be elucidated. As, however, not only shivering but also peripheral vasoconstriction and inhibition of polypneic panting could be obtained as effects of stimulation at the same points it might be possible that an integrative action on all mechanisms concerned with heat conservation is exerted from this part of the forebrain.

An exposure of the animals to heat with subsequent mobilization of heat loss mechanisms, or a rise in body temperature obtained by earlier effective stimulations did markedly diminish or abolish the shivering response to new stimulations. On the

other hand, the stimulations did cause a marked reduction of the respiratory rate in a panting animal and an exposure of the goats to cold or a lowering of the body temperature did facilitate the shivering response. The experiments thus give a demonstration of the balance between the central control of mechanisms concerned with the defense against heat and of those concerned with the defense against cold. It is in this connection interesting to notice a difference in the degree of thermoregulatory effects of stimulation of the preoptic "heat loss centre" and of the septal area in the goat. It has been shown in another series of experiments (ANDERSSON and PERSSON, in preparation) that it is possible to maintain polypneic panting and peripheral vasodilatation and to suppress shivering for more than two hours by continuous electrical stimulation of the "heat loss centre" in goats kept in an environmental temperature of -6°C . In this way the rectal temperature of the goats could be lowered as much as 10°C . The shivering response to stimulation in the septal area did, however, not persist when the rectal temperature had risen about $\frac{1}{2}^{\circ}\text{C}$, and the inhibitory effect of the stimulation on polypnea caused by the exposure to heat was markedly decreased when the rectal temperature had risen 1 to $1\frac{1}{2}^{\circ}\text{C}$ (Fig. 4). This difference might indicate some dominance of the preoptic "heat loss centre" over the structural elements concerned with the regulation against cold.

Summary.

Electrical stimulations in, or in the vicinity of the septum pellucidum of unanaesthetized goats were observed to produce shivering, peripheral vasoconstriction, sometimes piloerection and in animals exposed to heat an inhibition of polypneic panting.

Prolonged stimulation in room temperature caused a rise in rectal temperature of up to 0.5°C , at which stage shivering was no longer seen as result of stimulation.

A lowering of the body temperature of the animals, or the exposure to cold (4°C) facilitated the shivering response.

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Species Differences of Clotting Factors in Ox, Dog, Horse, and Man.

Prothrombin.

By

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The prothrombin content of plasmas from different species has been studied by several investigators with both one-stage and two-stage procedures. The results of these studies vary considerably, and they might be criticized for several reasons. The one-stage methods were unsuitable, since the results were influenced by all the conversion factors. In the two-stage methods an adequate supply of conversion factors in the conversion mixture has been disregarded, since factors have either been lacking or originated from a different species than did the prothrombin.

The purpose of the present work was a comparative study of the prothrombin activity in ox, dog, horse, and man taking these factors into account, and to investigate the possibility that a one-stage method, the Russell's viper venom-cephalin method of HJORT, RAPAPORT and OWREN (1955), could be used as a method for comparative investigations of prothrombin activity. This would imply a non species specificity of the different prothrombins towards Russell's viper venom.

Materials.

Saline brain thromboplastin was prepared as described by HJORT (1957, p. 17).

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Proconvertin was made from serum after clotting the plasma with 3 ml/100 ml of thromboplastin under vigorous stirring. After storage for 2 days at 20° C, proconvertin was prepared by the method of DUCKERT et al. (1953). Besides proconvertin, this preparation is also supposed to contain the Stuart factor (GRAHAM and HOGGIE 1956) and the Prower factor (BERGSAGEL 1955), and the preparation was therefore used as a source of these factors in some of the experiments.

Prothrombin was made from oxalated ox and dog plasma following the same procedure as for proconvertin. The bovine preparation was contaminated with proaccelerin since it was completely converted to thrombin after four minutes with homologous thromboplastin and calcium. With the dog preparation no thrombin was detected in 15 minutes after the addition of homologous thromboplastin and calcium. All adsorbable factors, of course, were present in the preparation.

Proaccelerin was prepared from bovine and dog plasma as described by HJORT (1957, p. 102).

Cephalin. The acetone insoluble, ether soluble fraction of human and bovine brain was prepared as described by HJORT, RAPAPORT and OWREN (1955).

Fibrinogen was prepared by the ether precipitation method of KEKWICK and MACKAY (1954). Bovine plasma was used, and the preparation was dissolved and diluted to optimal concentration in veronal buffer.

Blood was drawn in one tenth volume of either 3.13 per cent (w/v) trisodium citrate dihydrate or 2.5 per cent (w/v) potassium oxalate monohydrate. It was centrifuged for 30 minutes at 2,500 rpm in an International refrigerated centrifuge, model PR-2. Only clean venepunctures were accepted, and the first few milliliters were discarded. Usually the plasmas were used fresh. If not, they were kept in the deep freeze at -20° C.

Proaccelerin deficient citrated plasma was obtained from a patient with parahemophilia (OWREN 1947).

Adsorbed plasma. Oxalated plasma was adsorbed with 100 mg per ml of barium sulphate (Baker) for five minutes at 20° C.

Proconvertin deficient citrated plasma was obtained from a patient with congenital defect of this factor. The properties of this plasma has been described by OWREN (1952).

Veronal buffer (OWREN 1947). Sodium diethyl barbiturate 11.75 g, sodium chloride 14.67 g and 430 ml 0.1 N hydrochloric acid were mixed, and distilled water added to 2,000 ml (pH 7.35, ionic strength 0.154).

Dilution fluid I. One sixth volume of 0.1 M trisodium citrate dihydrate in 0.9 per cent (w/v) sodium chloride solution.

Dilution fluid II.

25.66 mM trisodium citrate dihydrate	200 ml
Veronal buffer	200 "
0.9 per cent (w/v) sodium chloride	600 "

Russell's viper venom. The preparation "Stypven", Burroughs Wellcome & Company, Inc., was used.

Methods.

Russell's viper venom (RVV)-cephalin method for prothrombin assay was carried out as described by HJORT, RAPAPORT and OWREN (1955), except that the substrate plasma was adsorbed, bovine plasma dialyzed against saline to remove the oxalate. In some experiments the human cephalin preparation was substituted with bovine cephalin.

Three-stage method for prothrombin assay (STORMORKEN and HJORT 1957). This method is a modified two-stage method with purified reagents.

In the *first stage* equal parts of thromboplastin, purified proconvertin, purified proaccelerin and calcium (10 mM) are incubated for 5 minutes at 37° C. Due to traces of prothrombin in the proconvertin preparation small amounts of thrombin form which convert the proaccelerin to accelerin. This stage, thus, results in the formation of prothrombinase.

In the *second stage* the prothrombinase formed in stage I converts the prothrombin to thrombin. The plasma to be assayed is diluted 1:5 in dilution fluid I, and further 1:10 in dilution fluid II to give a final dilution of 1:50. Of the dilute plasma, 0.4 ml is incubated in the water bath and recalcified with 0.4 ml 20 mM CaCl_2 , preheated to 37° C, just before the addition of 0.4 ml of the prothrombinase.

In the *third stage* the thrombin evolved is measured by adding 0.2 ml of the conversion mixture to 0.4 ml preincubated, purified bovine fibrinogen.

The first step was introduced to secure a rapid and fairly constant conversion of prothrombin. Homologous thromboplastin and proconvertin were used for assay in different species, but the proaccelerin was of bovine origin throughout.

Clotting times were measured in seconds and represent the mean of at least two determinations.

Experiments.

I. Investigations to establish the conditions necessary for the validity of two-stage methods in comparative studies.

1) The influence of homologous and heterologous thromboplastin.

To show the influence of the origin of thromboplastin on the peak thrombin activity, a usual two-stage system with bovine plasma was used, and the activation curve was followed both with bovine and human brain thromboplastin of optimal concentration. One ml of citrated bovine plasma diluted 1:50 in dilution fluid II was mixed with one ml of the thromboplastin suspension (bovine or human), and recalcified with one ml 25 mM of calcium chloride. At intervals, 0.2 ml were removed and tested

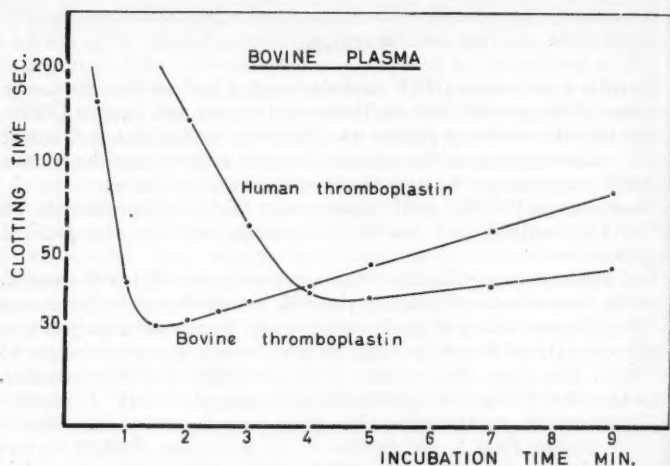


Fig. 1. Comparison of the effect of human and bovine thromboplastin in a two-stage assay of prothrombin in bovine plasma.

for thrombin activity in 0.4 ml preincubated, optimal dilution of bovine fibrinogen. The results are shown in figure 1. It is clear that the activation curves and the peak level differed for the two thromboplastins despite the concentrations were optimal. In two-stage methods in which the peak activity is used as an indicator of the prothrombin concentration, therefore, homologous thromboplastin must be used in the activation mixture.

2) *The influence of homologous and heterologous proconvertin.* It is now generally agreed that proconvertin should be present in optimal amounts in the two-stage prothrombin methods which are based on the peak activity. However, since we know from the work of MANN and HURN (1952) and from own experiments (to be published) that the reaction between proconvertin, thromboplastin and calcium to form convertin is very sensitive to species specificity, it was reasonable to assume that only homologous proconvertin could be used. The following experiment corroborated this assumption.

Plasma from a patient with congenital lack of proconvertin was activated with human thromboplastin, a) without proconvertin, b) with human proconvertin, and c) with bovine proconvertin added to the incubation mixture. The bovine proconvertin prep-

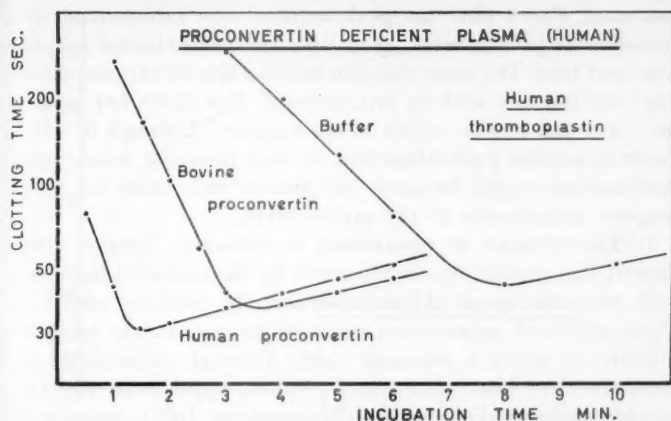


Fig. 2. Comparison between the effect of human and bovine proconvertin in a two-stage assay of prothrombin in proconvertin deficient human plasma using homologous thromboplastin.

aration was five times more concentrated than the human preparation. Its high activity was demonstrated by incubating equal amounts of bovine proconvertin, bovine thromboplastin, and calcium chloride 7.5 mM for two minutes at 37° C. By the simultaneous addition of 0.2 ml of this incubation mixture and 0.2 ml calcium chloride 25 mM to citrated bovine plasma, clotting occurred in 7 seconds.

The experiment was carried out as follows. The proconvertin deficient plasma was diluted 1:50 in dilution fluid II which contained 2 per cent of adsorbed bovine plasma. In this manner, a sufficient proaccelerin concentration was obtained to give a reasonable speed of conversion. One ml of this plasma dilution was mixed with 0.8 ml of human brain thromboplastin and 0.2 ml buffer, human proconvertin or bovine proconvertin respectively, and recalcified with one ml of 25 mM calcium chloride. At intervals, 0.2 ml were removed and tested for thrombin activity in 0.4 ml of preincubated, optimal dilution of bovine fibrinogen. The results are shown in figure 2. Comparison of the curves with and without proconvertin makes it clear that proconvertin is necessary for maximal thrombin activity. The middle curve, which represents the activation curve when bovine proconvertin

was used, shows that the peak activity was submaximal with heterologous proconvertin, even with the concentrated preparation used here. The same was also found when ox thromboplastin was used together with ox proconvertin. This curve ran between the lower and middle curves in the diagram. Although it will be shown in another publication that, in some instances, heterologous combinations might be used, the general rule must be to use reagents homologous to the prothrombin.

3) *The influence of proaccelerin or accelerin.* OWREN (1947) showed that proaccelerin is important for the thrombin formation, both when the speed of formation and the yield are concerned. Thus, sufficient proaccelerin must be present in the activation mixture to secure a maximal yield. Although proaccelerin and accelerin have been shown to be species specific on the basis of physicochemical properties (STORMORKEN 1957), experiments failed to demonstrate their specificity in activity. It is probable that quantity might compensate for quality in this case. Anyhow, it was found that the species origin did not influence the results, provided sufficient amounts of proaccelerin or accelerin were added to the conversion mixture. Owing to the stability and abundance of proaccelerin in bovine plasma, preparations from this species were used in this study.

4) *Other factors possibly influencing the conversion of prothrombin.* Little is known about the effect of factors other than those mentioned on the conversion of prothrombin by the tissue thromboplastin system. Both the Prower factor and the Stuart factor are said to influence the thromboplastin time (BERGSAGEL 1955, GRAHAM and HOUGIE 1956). However, since these factors are adsorbable, they were present in the proconvertin preparation, and a further consideration of these factors has therefore been disregarded.

5) *The influence of antithrombin.* The antithrombin is variable both within the same species and among different species, and this might affect the results. In the method used in this work, the influence of antithrombin has been reduced in three ways, viz. 1) by using purified reagents, 2) by a high dilution of the test plasma, 3) by a rapid and rather constant conversion time. The latter was obtained by changing the two-stage method into a three-stage, the first stage being the production of a strong prothrombinase.

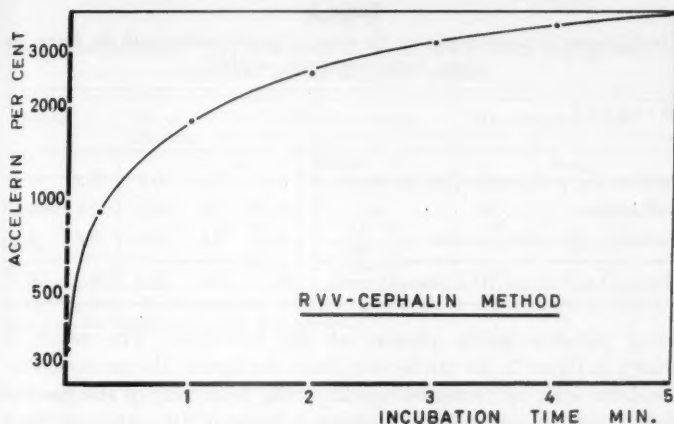


Fig. 3. The formation of accelerin in the substrate plasma of the Russell's viper venom-cephalin method during incubation before recalcification.

II. The use of the Russell's viper venom-cephalin method in comparative investigations.

1) *The influence of proaccelerin or accelerin.* In this method both thromboplastin and proconvertin are circumvented. Influences from these factors due to species differences may therefore be disregarded. Proaccelerin, on the other hand, has a profound effect on the conversion of prothrombin by the Russell's viper venom. Since the concentration of proaccelerin varies markedly among species, this variation might invalidate the comparison of species differences in prothrombin activity, although large amounts of proaccelerin are supplied in the test by using adsorbed bovine plasma as the substrate plasma. HJORT (1957 p. 119) has shown that proaccelerin is readily activated to accelerin by Russell's viper venom, even in the absence of calcium. In the following experiment the formation of accelerin during incubation of adsorbed ox plasma and the Russell's viper venom-cephalin reagent was studied. Equal parts of adsorbed ox plasma, RVV-cephalin reagent, and citrated dilution fluid II were mixed and incubated at 37° C. This imitated the original test except that the dilute plasma was substituted with the dilution fluid. At intervals, aliquots were removed and tested for proaccelerin-accelerlin activity with the one-stage proaccelerin assay method

Table 1.

The influence of proaccelerin on the assay of prothrombin with the Russell's viper venom-cephalin method.

Reagent	ml				
Purified dog proaccelerin (500 per cent) .	0.0	0.1	0.2	0.3	0.4
Buffer-saline	0.4	0.3	0.2	0.1	0.0
Purified prothrombin solution	0.2	0.2	0.2	0.2	0.2
Clotting times in the RVV-cephalin meth.	56.3	55.8	55.8	56.2	56.0

using parahemophilia plasma as the substrate. The result is shown in figure 3. As can be seen from the figure, the proaccelerin-activator activity increased rapidly at the beginning of the incubation, then a gradually diminishing increase per unit of time followed. In the course of the prescribed three minutes' incubation period before recalcification, the activity increased tenfold, reaching 3,000 per cent, or about 30 times the normal human plasma activity. Thus, there is no reason to believe that the proaccelerin concentration of the test plasma would influence the results. This was corroborated by the following experiment. Purified dog prothrombin, nearly free of proaccelerin, was mixed with increasing amounts of purified dog proaccelerin as shown in table 1. The preparation contained 500 per cent proaccelerin activity as measured in the one-stage proaccelerin assay using parahemophilia plasma as the substrate. The mixture was assayed without further dilution with the RVV-cephalin method. As can be seen from table 1, the increase in proaccelerin concentration of the prothrombin mixture did not influence the results.

2) *Comparison of the results of prothrombin estimation in different species with the three-stage method and with the RVV-cephalin method.* In ten plasmas from each of ox, dog, and horse the prothrombin activity was estimated with both the RVV-cephalin method and the three-stage method. In the latter, the reagents were homologous except for proaccelerin and fibrinogen, which were of bovine origin. The standard curve was made by assaying different dilutions a mixture of ten normal human plasmas, and therefore the figures are relative to the prothrombin activity in normal human plasma. The results are shown in table 2. Firstly, it is seen from the results with the three-stage method that the prothrombin activity did not vary appreciably in these species.

Table 2.

The "concentrations" of prothrombin in ox, horse, and dog plasma as estimated by two different methods.

Species	No. of animals	RVV-cephalin method		Three-stage method	
		\bar{m}	Range	\bar{m}	Range
Human	10	100	—	100	—
Ox	10	49	44—59	86	78—103
Horse	10	71	66—79	99	89—105
Dog	10	123	115—137	80	72—90

The figures refer to per cent activity in relation to that of human, and are corrected for variations in the hematocrit.

Secondly, a pronounced discrepancy between the two methods is obvious, the RVV-cephalin method giving considerably lower values in ox and horse plasma than the three-stage method, whereas the inverse is true for dog plasma. This finding has two implications, 1) the RVV-cephalin method cannot be used in its original form for comparative studies, 2) a species specificity of prothrombin or some factor(s) influencing the activity of Russell's viper venom must exist. However, the results of the two methods for individual samples within the same species corresponded excellently (see table 3). This applied to all species tested. The figures in table 3 represent the first five samples in the bovine series in table 2. To facilitate the comparison between the results of the two methods the first figure for the RVV-cephalin method was given the same value as the corresponding figure for the three-stage method, and the other figures were calculated in relation to this. The figures show that although the two methods give quite different absolute values, they reveal variations within the same species with closely corresponding results.

3) *Studies on the discrepancy between the results with the RVV-cephalin method and the three-stage method in comparative investigations.* At least three factors might be responsible for the discrepancy between the two methods in comparing prothrombin activity in different species, 1) the species origin of the cephalin preparation used in the RVV-cephalin method, 2) a species difference in some factor or factors affecting the activity of the Russell's viper venom, 3) a species difference in the reactivity of prothrombin towards Russell's viper venom, *i. e.* a chemical difference of the prothrombin in different species.

Table 3.

The correspondence of individual results within the same species for the Russell's viper venom-cephalin method and the three-stage method.

Sample No.	RVV-cephalin method		Three-stage method	
	Estimated per cent	Calculated, relative to first figure for three-stage	Estimated per cent	Deviation
1	59	103	103	0
2	45	79	76	+ 3
3	49	86	88	- 2
4	45	79	83	- 4
5	48	84	82	+ 2

To test the first possibility, the experiment illustrated in table 4 was performed. Plasmas from different species were estimated for prothrombin activity with the RVV-cephalin method using cephalin preparations of human and bovine origin. It is seen from the table that the results in all species corresponded closely with the two cephalin preparations. It might therefore be concluded that the origin of the cephalin is not the cause of the discrepancy between the methods. Although only two different cephalins were tested, this conclusion seems valid because any difference would have been revealed in one or more of the four different plasmas.

In the RVV-cephalin method, the test plasma is the only source of the adsorbable factors. According to recent literature, one or more of these factors affect the activity of Russell's viper venom (BERGSAGEL 1955, HOUGIE 1956). Thus, the possibility existed that species differences in these factors could account for the discrepancy between the two methods. This was investigated by comparing the results with and without the addition to the RVV-cephalin system of a proconvertin preparation from the different species studied here. As an example, the results with the two most deviating plasmas, *i. e.* ox and dog plasma, are presented in table 5. The preparations were added undiluted in 0.1 ml amounts to the RVV-cephalin system, and buffer was used as the blind. A preparation from dog was used in the experiment in table 5. As can be seen, some shortening of the clotting times was obtained in both plasmas by addition of the adsorbate, and somewhat more with the bovine plasma than with the dog plasma. To have the difference between the methods abolished,

Table 4.

Comparison between the results of prothrombin estimations in different species using the Russell's viper venom method with cephalin of human and bovine origin.

Plasma species	Source of cephalin in the RVV-cephalin method			
	Human		Bovine	
	Clotting time	per cent	Clotting time	per cent
Human	31.7	90	30.0	93
Ox	50.1	42	48.5	45
Horse	35.3	76	33.5	80
Dog	25.1	131	24.3	135

Clotting times in seconds.

however, the clotting time for the bovine plasma should have been shorter than that of the dog plasma, since its prothrombin content was higher as measured with the three-stage method. This finding makes it unlikely that the adsorbable serum factors are responsible for the difference between the RVV-cephalin and three-stage method.

The third possibility was investigated by the experiment illustrated in figure 4. Purified prothrombin from ox and dog were activated with a Russell's viper venom-cephalin mixture and a constant amount of proaccelerin, and the evolution of thrombin was examined at frequent intervals. The prothrombin preparations were adjusted to the same activity with the three-stage method, and the incubation mixture consisted of 0.4 ml prothrombin solution, 0.4 ml purified bovine proaccelerin, and 0.4 ml of the RVV-cephalin reagent which was a one to ten dilution in buffer of the ordinary reagent. This mixture was incubated for 3 minutes to have the proaccelerin converted to accelerin. The mixture was then recalcified to a final concentration of 2.5 mM, and at intervals of 10 seconds 0.2 ml were removed and tested for thrombin activity in 0.4 ml of purified fibrinogen. As figure 5 shows, the dog prothrombin preparation was activated considerably faster than was the bovine preparation. While the dog preparation was completely converted in 30 seconds, more than 60 seconds were required for the bovine. The fibrinogen contained no anticoagulant, and this is the reason why some thrombin activity was found even at zero time because conversion was

Table 5.

The effect on the results with ox and dog plasma of adding to the RVV-cephalin test a preparation adsorbed from dog serum.

	Bovine plasma		Dog plasma	
	Clotting time	per cent	Clotting time	per cent
RVV-cephalin method				
With buffer	46.5	48	25.6	124
With adsorbate . .	37.8	68	24.0	136
Three-stage method.	32.5	97	38.8	78

Clotting times in seconds.

possible in the test system. It is also seen that the conversion curves are straight lines nearly up to complete conversion. This is caused by the production of accelerin in the preincubation period whereby the basis for the autocatalytic type of curve was removed.

The same results as above were found also when the cephalin reagent in the RVV-cephalin preparation was of bovine origin, or when a preparation adsorbed from serum of different species was added to the system. It seems, therefore, that the discrepancy between the RVV-cephalin method and the three-stage method in comparative investigations is caused by a species difference in reactivity of prothrombin to Russell's viper venom, suggesting that prothrombin is chemically different in different species.

Discussion.

In earlier investigations on prothrombin concentration in various mammals, the specificity of the conversion factors has not been considered. WARNER, BRINKHOUS, and SMITH (1939) tried different thromboplastins, but found that this did not influence the results. This is contradictory to the present findings, and the reason for this is not known.

In table 6 are compiled the earlier investigations of prothrombin activity in the species concerned in this paper. There are great variations between the results of different workers. It is probable that these variations may chiefly be explained on basis of species specificity of the converting factors which have not been

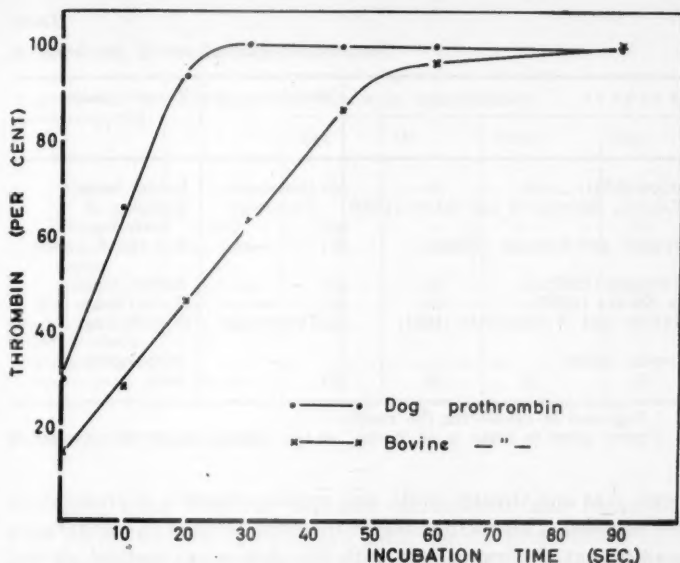


Fig. 4. The effect of Russell's viper venom on dog and bovine prothrombin. Dog and bovine purified prothrombin were activated with the same mixture of RVV-cephalin and proaccelerin, and the evolution of thrombin followed. The curves represent the mean of four experiments.

considered. Most of the results are obtained with bovine fibrinogen and the variations cannot therefore be explained by the specificity of the thrombin fibrinogen reaction. The figures coming closest to the present ones are those of Marbet and Winterstein, although they did not consider the species specificity of the conversion factors. This might be explained by the introduction of a first stage in their method, in which thromboplastin, proconvertin, proaccelerin and calcium are allowed to form prothrombinase. This lessens the influence of heterologous reagents, but does not abolish it as will be shown in a subsequent publication. As compared with our values, their figure for ox is higher, the others lower. The explanation probably is that they used bovine proconvertin throughout which favoured the conversion of the bovine prothrombin.

QUICK's one-stage method cannot be used to measure prothrombin separately as already pointed out by WARNER, BRINKHOUS and SMITH (1936). In the RVV-cephalin method, however, the thromboplastin-proconvertin reaction is circumvented (RAPA-

Table
Comparative estimations of prothrombin

Authors	Method	Thromboplastin
QUICK (1941)	One-stage	Rabbit brain
WARNER, BRINKHOUS and SMITH (1939)	Two-stage	Homolog. & heterolog.
MURPHY and SEEGER (1948)	— * —	Not stated
SCHULTZE (1949)	— * —	Rabbit brain
DE NICOLA (1953)	— * —	Rabbit lung
MARBET and WINTERSTEIN (1954)	Three-stage	Rabbit lung
Present author	— * —	Homologous brain

¹ Regarded in calculating the results.

Figures given in units or as "index" in the original papers are converted to

PORT, AAS and OWREN 1954), and surplus amounts of proaccelerin are supplied. This method might therefore be used in comparative studies, but the comparison with the three-stage method showed that it could not. This finding is therefore in conflict with the statement that Russell's viper venom possesses no group specificity in contradistinction to tissue thromboplastin (QUICK 1945). However, as seen in table 3, within the same species both methods reflect the individual fluctuations identically. Due to its far more practical applicability, therefore, the RVV-cephalin method might be used with advantage in other species than man provided the results are read from a standard curve obtained with the species concerned.

Recent work have revealed serum factors which affect the activity of Russell's viper venom. This seems to be established concerning the Stuart factor (GRAHAM and HOGGIE 1956, HOGGIE 1956), whereas the statements are conflicting as to the Prower factor (BERGSAGEL 1955, TELFER et al. 1956). It was possible that the discrepancy between the RVV-cephalin method and the three-stage method observed in table 2 was caused by a species variation of these factors. Both the experiments with plasma and with purified reagents show that, although the proportion between the figures changed, the serum factors could not account for the discrepancy. In an experiment parallel to that reported in figure 4 all conditions favoured the conversion of bovine pro-

Table
Thrombin

6.

in man, ox, horse, and dog.

Conversion factors supplied	Fibrinogen	Prothrombin "concentration"			
		Man	Ox	Horse	Dog
None	—	100	80	200	500
None	Dog	100	—	—	118
Adsorbed ox serum	Not stated	100	88	—	69
None ¹	Bovine	100	89	150	140
Not stated	Not stated	100	90	—	50
Bovine proconv. and accelerin	Not stated	100	100	90	70
Homolog. proconv. bovine pacc.	Bovine	100	86	99	80

per cent by the present author.

thrombin, since both the cephalin, proaccelerin and fibrinogen were of bovine origin. Still, the prothrombin preparation from dog was converted much faster. On the basis of these experiments it seems justifiable to conclude that the chemistry of prothrombin varies between the species.

After the establishment of the specificity of the thrombin fibrinogen reaction (STORMORKEN 1957), the comparison of prothrombin concentration between species has become fictitious. Such a comparison deals only with prothrombin *activity* as measured in an arbitrarily chosen test system, *e. g.* in this case bovine fibrinogen. The activity measured in various species is related to, but not proportional to the true prothrombin concentration. In the present case the figures for the ox species are relatively too high because the thrombin prefers the homologous fibrinogen. If dog fibrinogen were chosen, the figures for dog would be relatively too high, and the figures for the other species would deviate from those with bovine fibrinogen. The variation in the results of comparative prothrombin estimations by different markers might partly be due to the species specificity of the thrombin fibrinogen reaction since different fibrinogens have been used. So far, correction for the species specificity of the indicator system is impossible since there is no fixed point of reference. However, as can be seen from the paper mentioned above, this point is far from negligible.

Summary and Conclusions.

- 1) In one-stage or two-stage methods for prothrombin assay using tissue thromboplastin, the proconvertin (factor VII, SPCA) and thromboplastin should be homologous with the prothrombin.
- 2) Due to the species specificity of the thrombin-fibrinogen reaction a comparison of prothrombin *concentrations* between species is fictitious. Plasmas from different species can only be compared as to prothrombin *activity* in a defined test system.
- 3) With a modified two-stage method using homologous reagents except for proaccelerin and fibrinogen, which were of bovine origin, prothrombin activity was estimated as follows: Man 100, ox 86, horse 99, and dog 80 per cent.
- 4) Within each of the species tested, the Russell's viper venom-cephalin method reflected individual fluctuations in close conformity with the modified two-stage method. It is therefore recommended as a routine method.
- 5) In comparing species, discrepancies between the two methods were disclosed. These were caused by a varying effect of Russell's viper venom on prothrombin from different species, indicating species differences in the chemistry of prothrombin.

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Supersensitivity Caused by Denervation and by Cholinesterase Inhibitors.

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Several theories have been advanced regarding the mechanism of the increased sensitivity to chemical agents that develops after denervation (for ref. see CANNON and ROSENBLUETH 1949). One hypothesis that seemed plausible and that also seemed to lend itself to investigation is the "enzyme-theory", according to which the increased sensitivity to drugs is due to a decreased activity of inactivating enzymes in the denervated organ as compared with the normally innervated organ.

In previous experiments (STRÖMBLAD 1955 b) it was found that the activity of acetylcholine splitting enzymes in the parotid gland of the cat was diminished two weeks after parasympathetic denervation of the gland. It was further found that postganglionic denervation of the gland caused a greater decrease in enzymic activity and a greater degree of supersensitivity than preganglionic denervation. The submaxillary gland was also used in the previous experiments. The results obtained with this gland were difficult to interpret since the parasympathetic denervation of this gland causes a considerable atrophy of the gland; the enzymic activity was found to decrease when calculated per gland, but no difference could be found when the enzymic activity was calculated per gram of tissue.

The demonstration of a fall in enzymic activity *in vitro* is not sufficient to establish a cause-relationship between a decrease in enzymic activity and a supersensitivity, since it is known that the activity of cholinesterases may be inhibited to a certain

degree without any effect on the response to the drugs splitted by the enzymes. The degree of inhibition necessary to alter the response to these drugs has been estimated by several investigators and the figures arrived at differ for the various organs (for discussion see SHELLEY, 1955). No investigation on the parotid gland of the cat has been found and it thus seemed of interest to use this gland. There are two investigations of this type on the sub-maxillary gland; RIKER and WESCOE (1949) found that a decrease by 50 % was necessary to affect the sensitivity, while the figures of DIRNHUBER and EVANS (1954) suggest that a less decrease may be sufficient.

Even if now a decrease of a sufficient degree in an extract of the organ thus determined could be demonstrated after denervation, the importance of the fall in enzymic activity after denervation is not clear. It might well be that there is enzyme in different locations; the enzyme in certain locations being of importance for the destruction of administered drugs, the enzyme in other locations being of no importance for injected drugs. To get some information concerning this problem both the crude homogenate and the supernatant of the homogenate obtained after low speed centrifugation was used for estimation of cholinesterase activity, presuming that enzyme differently located could possibly behave differently to this procedure.

Methods.

Cats were used for the experiments.

The denervations were made under nembutal anaesthesia (30 mg/kg intraperitoneally) using the same technique as described previously (STRÖMBLAD 1955). Two weeks later, when not otherwise stated, the acute experiment was made. Intravenous chloralose anaesthesia (about 80 mg/kg) after preliminary ether was used. The salivary ducts were exposed and glass cannulae inserted. The other end of the cannulae was connected to a piece of rubber, which in turn was connected to a piece of glass.

Cholinesterase inhibitors, mintakol¹ (paraoxon) or TEPP¹, were administered to the glands via the excretory ducts using the technic of EMMELIN, MUREN and STRÖMBLAD (1954). The rubber piece was occluded by a pair of artery forceps and the injection (0.1 cc) made into the rubber piece central to the occlusion. The drugs were dissolved in saline and the injection made during 5 sec. After another 5 sec. the occluding forceps were removed.

¹ My thanks are due to docent B. HOLMSTEDT, Stockholm, for kindly supplying the drugs.

Drugs, other than the enzyme inhibitors, were given intravenously via a cannula inserted into a femoral vein. The drops of saliva falling from the cannula were signalled on the smoked drum and counted.

At the end of an experiment, before the glands were removed for estimation of enzymic activity, a fairly big dose of methacholine was given, so as to wash out any enzyme inhibitor still left in the ducts of the gland. This washing out was found not to interfere with the sensitivity of the gland, since a small dose of acetylcholine before and after the big dose of methacholine gave the same secretory response.

The animal was then killed by air embolism and the glands removed, carefully cleaned, washed between filter papers and weighed. The glands were cut with scissors and ground in a glass homogenator together with Krebs' bicarbonate-Ringer. The volume in ml of Krebs' bicarbonate-Ringer was for the parotid glands 4 times and for the submaxillaries 8 times the weight of the gland in gram. The homogenate was then stored at -20°C to the next day.

The estimations of cholinesterase activity were made manometrically (AMMON 1933) using Warburg technique. The main compartment contained the enzyme sample and the volume was brought up to 1.7 ml with Krebs' bicarbonate-Ringer. The side bulb contained the substrate in a volume of 0.3 ml. The enzyme was either homogenate (0.2 ml) or the supernatant (0.3 ml) of the homogenate after centrifugation at 3,000 rev. p. m. for 15 min. The substrates used were the chlorides of acetylcholine (ACh), methacholine¹ (MCh) and butyrylcholine (BuCh). The final concentrations were 0.011 M (0.2 %), 0.035 M (0.6 %) and 0.029 M (0.6 %), respectively.

The flasks were filled with nitrogen containing 5 % CO_2 and incubated at 37°C . Readings were taken every 6 min. for 42 min. In all experiments a thermobarometer and an "enzyme blank" were used and corrections for changes in these flasks were made. The carbon dioxide evolution was plotted on a graph against the time and the reading for 30 min. was calculated and corrected for non-enzymic hydrolysis of the substrate.

The values obtained were expressed as $\mu\text{l CO}_2$ evolved/g of tissue/30 min. = b_{30} (AUGUSTINSSON 1948) or as $\mu\text{l CO}_2$ evolved/gland/30 min. = total.

All estimations were made in duplicate.

Results.

I.

In the earlier work (STRÖMBLAD 1955 b) on cholinesterase activity of the salivary glands of the cat after parasympathetic denervation the enzymic activity was estimated only in the supernatant of the homogenate. As mentioned above it seemed to be of interest

¹ My thanks are due to the firm Erik Lindblom & Co, Stockholm, for kindly supplying the drug.

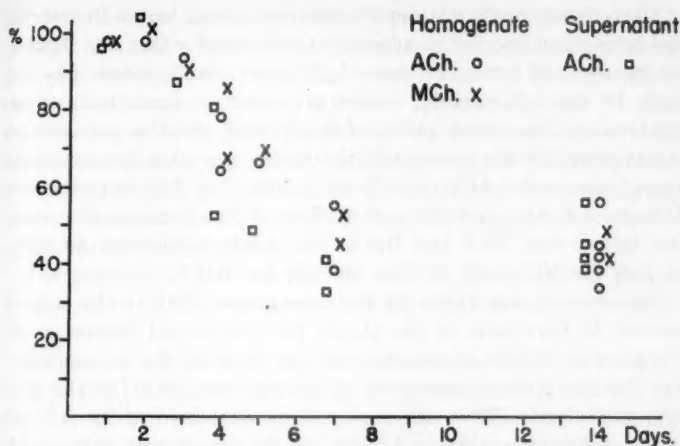


Fig. 1. The figure shows the activity of cholinesterase in the postganglionically parasympathetically denervated parotid gland in per cent of the contralateral normal gland of each animal. The total activity figures (vide methods) are used. Ordinate: per cent activity. Abscissa: time in days.

The enzyme sample and substrates used in the cholinesterase estimations are given in the figure.

to estimate different fractions of an extract of the glands. The crude homogenate was therefore used (as well as the supernatant) in a series of experiments to determine the effect of parasympathetic denervation. The results of the experiments with postganglionic parasympathetic denervation of the parotid gland are shown in fig. 1. On the ordinate is plotted the activity of the denervated gland in per cent of the contralateral normally innervated gland of each animal and on the abscissa the time in days after the denervation.

It can be seen that the activity of acetylcholine splitting enzyme of the homogenate and the supernatant are similarly affected by the denervation; the activity of methacholine splitting enzymes in the homogenates also closely follow that of the acetylcholine splitting ones.

The fall in enzymic activity, as can be seen in the figure, takes place on the 2nd to 7th day, and from then on it seems to be completed. SCHOFIELD (1952) working on the iris of the cat found that the decrease in cholinesterase activity after postganglionic denervation was completed in 3 weeks.

After parasympathetic denervation the gland loses in weight and thus when the fall in activity is calculated either the figures for gas evolved per g of tissue (b_{30}) or per gland (total) may be used. In the following b_{30} values are given in parenthesis after total values. The mean values of denervated gland in per cent of contralateral for the cats estimated two weeks after denervations were: homogenate ACh ($n = 5$) 43 ± 3.7 (48 ± 2.5); supernatant ACh ($n = 4$) 45 ± 4.9 (50 ± 2.4). Two of the homogenates were also tested with MCh and BuCh. The percentages were 49 (50); 41 (48) for MCh and 72 (73); 60 (70) for BuCh.

The activity was higher in the homogenate than in the supernatant. In four pairs of the glands just considered the mean of the activity of the supernatant in per cent of the homogenate was for the normal glands 82 ± 1.2 and 84 ± 4.2 for the denervated glands. Thus the supernatant contained about 4/5 of the acetylcholine splitting activity of the gland both in normal and denervated glands.

In 6 cats the preganglionic parasympathetic pathway to the parotid gland was severed and the enzymic activity of the glands estimated two weeks later. The mean values of the denervated gland in per cent of its contralateral gland were: homogenate ACh ($n = 6$) 72 ± 5.7 (89 ± 7.3), homogenate MCh ($n = 5$) 64 ± 5.5 (78 ± 4.2) and supernatant ACh ($n = 3$) 70 (80). The activity of the supernatant in per cent of the homogenate was both for denervated ($n = 3$) and for normal glands ($n = 3$) of this series 79.

Longcontinued treatment with an atropine-like substance causes a supersensitivity of the parotid gland (STRÖMBLAD 1956); it was also found that this treatment did not change the relation between the postganglionically denervated and the normal parotid gland when the acetylcholine splitting activity of the supernatant was estimated. In the present experiments two animals with the parotid gland of one side postganglionically denervated were treated during two weeks with Hoechst 9980¹ (piperidino-aethyl-diphenyl-azetamid hydrochloride, 1 mg/kg subcutaneously once a day). The acetylcholine splitting activity of the denervated gland in per cent of the normal were: homogenate ACh 49 (53) and 43 (53); supernatant ACh 45 (50) and 39 (58). The activity of the supernatant was in per cent of the homogenate (mean of

¹ My thanks are due to the firm Webass Ltd, Gothenburg, for kindly supplying the drug.

the two) for the denervated side 72 and for the normal side 77.

In 3 cats the preganglionic nerves to the submaxillary gland (chorda tympani) was cut. The mean values of denervated in per cent of normally innervated were: homogenate ACh 73 (99), supernatant ACh 83 (115). In one cat the MCh splitting activity of the homogenate was estimated. The activity of the denervated gland was 69 (81) per cent of the normal gland. For anatomical reasons it is not possible to make a postganglionic denervation of the submaxillary gland.

The activity of the supernatant was for the 3 normal glands 39 per cent of the homogenate and for the 3 denervated glands 32 per cent.

Besides by exclusion of parasympathetic impulses it is possible to sensitize the salivary glands by sympathetic denervation, *i. e.* excision of the superior cervical ganglion. This was made on 3 cats. The denervated in per cent of normal was for parotids: homogenate ACh 102 (94), supernatant ACh 103 (96) and for submaxillaries: homogenate ACh 104 (97), supernatant ACh 101 (95). The activity of the supernatant was for the denervated parotid glands 80 per cent of the homogenate and for the denervated submaxillaries 37.

The results so far reported could be summarized as follows: postganglionic parasympathetic denervation causes a decrease to about 40 (50) of the acetylcholine splitting activity of the homogenate and supernatant of the gland; a similar decrease was found for the homogenate when MCh was used as substrate. When BuCh was the substrate the decrease in the homogenate was less. Preganglionic denervation of this gland causes a decrease to about 70 (80) per cent of the ACh and MCh splitting enzymes.

Treatment with a parasympatholytic drug did not change the relation in acetylcholinesterase activity in homogenate or supernatant between the denervated and innervated gland.

Section of the chorda tympani caused a fall in the amount of enzyme present in the gland to about 75 per cent both in homogenate and supernatant, whereas the concentration was unchanged or raised.

Excision of the superior cervical ganglion did not alter the enzymic activity of the salivary glands either when the supernatant or when the homogenate was estimated with ACh as substrate.

The relation between supernatant and homogenate was not altered by any of the procedures, and thus there was no indication of different kinds of enzymes that were separated by low speed centrifugation.

Changes in enzymic activity in the supernatant after denervations and treatment with H₅ 9980 have been reported earlier (STRÖMBLAD, 1955 b and 1956) for another series of cats and the values here obtained agree with those previously reported.

II.

In section I the changes in cholinesterase activity caused by denervation were described. In the present section the changes in cholinesterase activity and sensitivity caused by cholinesterase inhibitors will be described, and these changes should be compared with those found after denervation.

The inhibitors used were mintakol and TEPP. TEPP was used only in a few experiments to support the assumption that the results obtained were due to inhibition of cholinesterase.

Increased sensitivity caused by inhibitors.

Parotid glands. 8 cats were used for these experiments. The sensitivity of the glands of the two sides to intravenously injected drugs was first tested. The drugs used for estimation of sensitivity were the same as later was used for estimation of enzymic activity in the particular animal. The doses of the drugs injected were chosen so as to get a small secretory response (1—2 drops of saliva). In this series of cats ACh was used in doses of 2 or 5 $\mu\text{g/kg}$; MCh 1 or 2 $\mu\text{g/kg}$ and BuCh in doses of 100 or 200 $\mu\text{g/kg}$. The drugs were injected at least two times each. The inhibitor was then injected into the right gland in a dose of 0.5 μg ; the corresponding volume of saline (0.1 cc) was injected into the contralateral duct. The sensitivity to the series of drugs was then estimated 10 min. after the injection of the inhibitor (it was found that the full effect of the inhibitor was not obtained until 10 min. after the injection).

In one experiment the glands were taken out after this single dose (cat no. 4) although no change in sensitivity attributable to the inhibitor was detectable. In other cases the dose was repeated or increased until there was a definitely increased sensitivity of the gland treated with inhibitor towards one or more

Table I.

Mintakol or TEPP (cats no. 1 and 7) injected into the right parotid duct. For each gland are given weight in gram, b_{30} (concentration) and total activity i. e. $\text{weight} \times b_{30}$. To the right of these are given the activity of right gland in per cent of the left gland of each animal both for b_{30} and total values. There are four figures given for each animal. From above the figures show: homogenate ACh, homogenate MCh, homogenate BuCh and supernatant ACh. To the far right is given the secretory response after the administration of inhibitor for each of the 3 drugs used for enzymic estimations. R = right gland; L = left gland.

Cat no.	Right gland			Left gland			Right gland in per cent of left gland		Secretory response
	weight in g	b_{30}	total	weight in g	b_{30}	total	b_{30}	total	
1	0.64	725	464	0.59	1,125	664	64	70	ACh R > L
		550	352		825	487	67	72	MCh R > L
		175	112		575	339	30	33	BuCh R > L
2	0.79	1,325	1,047	0.76	1,725	1,311	77	80	ACh R = L
		575	454		800	608	72	75	MCh R = L
		225	178		550	418	41	43	BuCh R > L
3	0.42	2,675	1,124	0.42	2,475	1,040	108	108	ACh R = L
		1,375	578		1,325	557	104	104	MCh R = L
		450	1,890		750	315	60	60	BuCh R > L
4	0.80	1,150	920	0.78	1,100	858	105	107	ACh R = L
		625	500		613	478	102	105	MCh R = L
		413	330		475	371	87	89	BuCh R = L
5	0.58	1,125	653	0.60	2,050	1,230	55	53	ACh R > L
		575	334		1,175	705	49	47	MCh R > L
		200	116		575	345	35	34	BuCh R > L
6	0.57	1,675	955	0.60	2,225	1,335	75	72	ACh R > L
		825	470		1,125	675	73	70	MCh R > L
		225	128		600	360	38	36	BuCh R > L
7	0.70	950	665	0.70	1,575	1,103	60	60	ACh R > L
		525	368		725	508	72	72	MCh R > L
		275	193		500	350	55	55	BuCh R > L
8	0.83	1,350	1,121	0.83	2,025	1,681	67	67	ACh R > L
		713	592		1,175	975	61	61	MCh R > L
		—	—		—	—	—	—	

Table II.

Mintakol injected into the right submaxillary duct. For further explanations see table I.

Cat no.	Right gland			Left gland			Right gland in per cent of left gland		Secretory response
	weight in g	b ₃₀	total	weight in g	b ₃₀	total	b ₃₀	total	
9	0.97	3,195	3,099	1.06	4,095	4,341	78	71	ACh R > L
		1,890	1,833		2,610	2,767	72	66	MCh R > L
		—	—		—	—	—	—	
10	1.36	1,935	2,632	1.40	2,993	4,190	65	63	ACh R > L
		878	1,194		1,305	1,827	67	65	MCh R > L
		518	704		878	1,229	59	57	BuCh R > L
		1,140	1,550		1,815	2,541	63	61	
11	0.65	2,475	1,609	0.64	4,140	2,650	60	51	ACh R > L
		1,001	651		1,553	994	64	65	MCh R > L
		653	424		1,080	691	60	61	BuCh R > L
		—	—		—	—	—	—	
12	1.39	2,138	2,972	1.37	2,115	2,898	101	103	ACh R = L
		1,080	1,501		1,080	1,480	100	101	MCh R = L
		338	470		585	801	58	59	BuCh R > L
		885	1,230		900	1,233	98	100	

of the drugs. It was consistently found that the secretory response to BuCh was affected by a lower dose of the inhibitors than the responses to the other drugs. It was often also found that the response to ACh could be altered before any changes in the response to MCh appeared. The doses of mintakol necessary to increase the response varied for different animals. Assuming full accumulation of the injected doses the amount varied between 1 and 7 μ g. In two cats of this series TEPP was used. The total amounts of this drug injected were 1.5 and 5 μ g (cat no. 1 and no. 7 respectively).

Table I gives the results of the estimations of enzymic activity and an indication to whether sensitization towards the different drugs were present or not.

From the table it may be deduced that a sensitization towards ACh and MCh was present when the ACh, respectively MCh, splitting activity of the sensitized gland was about 70 per cent

of the contralateral gland. The corresponding figure for BuCh is less well defined but it seems to be of the same order of magnitude.

Submaxillary glands. The outcome of the experiments on the submaxillary gland is shown in table II. The table seems to allow the conclusion that a decrease to 60–70 per cent of ACh, MCh and BuCh splitting enzymes of the gland causes a supersensitivity to the respective drugs.

It could be hypothesized that the inhibitory drug injected did not remain in the gland injected but diffused and reached the contralateral gland and that this gland therefore could not serve as an indicator to the state of the starting level of the treated gland.

To get information concerning this two control experiments were made. In one of the experiments the right submaxillary gland was removed at the start of the experiment. The remaining salivary glands were cannulated. The sensitivity to the secretory effect of ACh was determined. 50 μ g of mintakol, *i. e.* a big dose, was injected into one parotid gland. The sensitivity of the submaxillary gland and the not injected parotid gland was estimated again. No difference in sensitivity of these glands was found, nor showed the animal any general signs of intoxication. The left submaxillary gland was taken out 45 minutes after the injection of the inhibitor and the enzymic activity of the right and left submaxillary gland was determined. The values for the left gland in per cent of the right were: homogenate ACh 95 (95); homogenate MCh 105 (105); supernatant ACh 93 (93).

In the other experiment one parotid gland was removed before the experiment and the injections were made into a submaxillary gland (50 μ g of mintakol). In this case the percentage activity of the gland removed late in the experiment in terms of the previously removed were: homogenate ACh 97 (97); supernatant ACh 105 (105).

Thus there is no reason to believe that the doses used influenced the gland of the contralateral side.

It was also thought to be of interest to ascertain that the supersensitivity caused by mintakol did not decline rapidly. To test this the sensitivity of a parotid gland was tested 10 min. and 70 min. after the injection of the inhibitor. No difference in sensitivity was found. After 100 min. the sensitivity had declined.

Table III.

Left parotid gland postganglionically parasympathetic denervated 2 weeks earlier. Mintakol injected into the right parotid duct.

Cat no.	Right gland			Left gland			Right gland in per cent of leftgland		Secretory response
	weight in g	b ₃₀	total	weight b ₃₀	b ₃₀	total	b ₃₀	total	
13	0.86	963	828	0.72	1,550	1,116	62	74	ACh R > L
		573	441		725	522	71	84	MCh R = L
		225	194		425	306	53	63	BuCh R > L
		625	538		1,108	798	56	67	
14	1.06	650	689	1.02	1,050	1,071	62	64	ACh R > L
		300	318		438	447	68	71	MCh R > L
		200	212		375	383	53	55	BuCh R > L
		458	485		783	799	58	61	
15	0.69	525	362	0.65	825	536	64	68	ACh R > L
		242	167		350	228	69	73	MCh R < L
		108	75		375	244	29	31	BuCh R > L

Abolition of the difference in sensitivity between a denervated and a normal gland.

The results obtained in the previous part showed that the fall in enzymic activity after postganglionic parasympathetic denervation was greater than that causing an increased sensitivity after injection of esterase inhibitors. As to the preganglionic denervations the most to be said is that if the total figures are considered the decrease is of the same order of magnitude as that necessary to cause an enhanced response. To get more informations on the possible rôle played by decrease in enzymic activity it was in some experiments tried to inject the not denervated side with inhibitors until it was equal in sensitivity to its two weeks earlier denervated fellow gland.

Parotid glands. In 3 cats the left gland was postganglionically denervated. Mintakol was injected into the right gland. Just as in the experiments reported, it was found that the sensitivity to BuCh was affected by the least dose and it was also found that the difference between the two glands in sensitivity towards this drug was abolished at a lower dose than the sensitivity towards the other drugs.

Table IV.

Right chorda tympani cut 2 weeks earlier. Mintakol injected into the left submaxillary duct.

Cat no.	Right gland			Left gland			Right gland in per cent of left gland		Secretory response
	weight in g	b ₅₀	total	weight in g	b ₅₀	total	b ₅₀	total	
16	0.72	3,375	2,430	1.09	1,530	1,668	45	69	ACh R > L
		878	632		405	441	46	70	MCh R > L
		1,305	940		383	417	29	44	BuCh R > L
		2,760	1,987		1,170	1,275	42	64	
17	0.65	4,525	2,941	1.07	825	883	18	30	ACh R = L
		2,288	1,487		600	642	26	43	MCh R > L
		1,725	1,121		275	294	16	26	BuCh R = L
		—	—		—	—	—	—	
18	0.55	3,625	1,994	1.10	630	693	17	35	ACh R = L
		2,070	1,139		270	297	13	26	MCh R = L
		1,935	1,064		315	347	16	33	BuCh R < L
		1,325	729		267	294	20	40	

The results of the experiments are summarized in table III. The doses of mintakol used were 15, 12 and 10 μ g respectively.

It seems justified to draw the conclusion that in order to get the same degree of supersensitivity by injecting inhibitors as by postganglionic denervation a greater inhibition of enzymic activity is necessary than that caused by denervation.

Submaxillary glands. The same type of experiment as just described for the parotid glands was made also with the submaxillary gland, *i. e.* the chorda tympani was cut on the right side and two weeks afterwards the cat was anaesthetized and inhibitors were injected into the left gland until the sensitivity of that gland was the same as that of the right denervated gland. The results are given in table IV. In all 3 cats 20 μ g of mintakol was used.

It can be seen that in order to get the same sensitivity of the not denervated as in the denervated (preganglionically) gland, it is necessary to decrease the enzymic activity to a level considerably below that attained after denervation.

Table V.

Right parotid gland pre- and left parotid gland postganglionically parasympathetically denervated 2 weeks earlier. Mintakol injected into the right parotid duct.

Cat no.	Right gland			Left gland			Right gland in per cent of left gland		Secretory response
	weight in g	b ₃₀	total	weight in g	b ₃₀	total	b ₃₀	total	
19	0.84	1,125	945	0.82	1,513	1,241	74	76	ACh R > L MCh R > L
		525	441		800	656	66	67	
		750	630		1,117	916	67	69	
20	0.44	825	363	0.44	750	330	110	110	ACh R = L MCh R = L
		375	165		375	165	100	100	
		—	—		—	—	—	—	
21	0.98	700	686	0.98	675	662	104	104	ACh R = L MCh R = L
		400	392		383	375	104	105	
		625	613		583	571	107	107	
22	0.75	500	375	0.77	550	424	91	88	ACh R < L MCh R < L
		275	206		250	193	110	107	
		400	300		383	295	104	102	

Abolition of the difference in sensitivity between pre- and postganglionically denervated glands.

It was found that postganglionic denervation caused a more pronounced fall in enzymic activity than preganglionic denervation. It has also been found previously that the postganglionic denervation causes a more pronounced increase in sensitivity than preganglionic denervation. Could this difference be caused by the difference in enzymic activity? To get some information on this problem 4 cats were preganglionically denervated on one side and postganglionically denervated on the other side. The preganglionically denervated gland was at the acute experiment injected with enzyme inhibitor until the sensitivity of the two glands was equal. Table V gives the figures arrived at in these experiments. 10 μ g of mintakol was used in each of the 4 animals.

It seems to be justified to draw the conclusion that most of the difference between a pre- and a postganglionically denervated

Table VI.

Right and left parotid glands postganglionically parasympathetically denervated 2 weeks earlier. Mintakol injected into the right parotid duct.

Cat no.	Right gland			Left gland			Right gland in per cent of left gland		Secretory response
	weight in g	b ₃₀	total	weight in g	b ₃₀	total	b ₃₀	total	
23	0.54	375	203	0.52	500	260	75	78	ACh R > L MCh R > L
		175	95		225	117	78	81	
		300	162		367	191	82	85	
24	0.96	688	660	0.97	925	897	74	74	ACh R > L MCh R > L
		250	240		375	364	67	66	
		400	384		642	623	62	62	
25	0.67	525	352	0.66	825	545	64	65	ACh R > L MCh R > L
		250	168		350	231	71	73	
		475	318		617	407	77	78	
26	0.54	1,175	635	0.53	1,300	689	90	92	ACh R > L MCh R > L
		525	284		550	292	95	97	
		800	432		1,000	530	80	82	
27	0.74	750	555	0.70	1,100	770	68	72	ACh R > L MCh R > L
		300	222		463	324	65	69	
		400	296		683	478	59	62	
28	0.52	875	455	0.50	1,100	550	80	83	ACh R > L MCh R = L
		475	247		525	263	90	94	
		567	295		750	375	76	79	

gland in sensitivity to ACh and MCh is due to the greater fall in enzymic activity caused by the postganglionic denervation as compared with the preganglionic denervation.

Difference between denervated glands caused by inhibitors.

It is generally supposed that the enzyme in an organ is present in excess of the needs to destroy injected drugs. It was found for salivary glands that some 30 per cent had to be inhibited by mintakol before any increased sensitivity was found. If denervation acted on the same fraction of enzyme as mintakol, it would

Table VII.

Mintakol injected into the right and left parotid duct until increased sensitivity. Then further mintakol into the right duct.

Cat no.	Right gland			Left gland			Right gland in per cent of left gland		Secretory response
	weight in g	b ₃₀	total	weight in g	b ₃₀	total	b ₃₀	total	
29	1.23	525	646	1.21	650	787	81	82	ACh R > L MCh R = L
		213	262		225	272	95	96	
		—	—		—	—	—	—	
30	0.71	431	530	0.71	550	666	78	80	ACh R > L MCh R > L
		775	550		925	657	84	84	
		325	231		500	355	65	65	
31	1.43	—	—	1.43	—	—	—	—	ACh R > L MCh R > L
		575	822		775	1,108	74	74	
		375	536		475	679	79	79	
32	0.93	—	—	0.94	—	—	—	—	ACh R > L MCh R > L
		300	429		517	739	58	58	
		575	535		850	799	68	67	
		250	233		450	423	56	55	
		—	—		—	—	—	—	
		333	310		500	470	67	66	

mean that a less degree of inhibition would be necessary to affect the sensitivity of a denervated gland, since there was no longer any excess of enzyme to deal with. This presumption was tested by using 6 animals the parotid glands of which had been post-ganglionically denervated on both sides. The two glands of an animal were surprisingly equal in sensitivity after bilateral denervation, even though a slight difference could sometimes be found. The inhibitors were given to the right gland. The amounts of mintakol used varied between 10 and 30 μ g. Table VI gives the results of the experiments.

It can be seen that the percentage difference between the two fellow glands in this series was about the same or slightly less than in the case of normal glands when one gland was made supersensitivity by inhibitor. However, in the present series the starting values of enzymic activity can be considered to be only half of the normal value and the decrease in terms of the normal gland thus only half of the found value. This is supported by the following calculation.

If, instead of the percentage value for each pair, the difference between the two fellow glands is calculated, a lower figure is found in the series of double denervated glands than in the series of normal glands when a different sensitivity was caused by inhibitors. Thus for homogenate ACh the mean in the double denervated series is $227 \mu\text{l}/30 \text{ min}/\text{gram}$ while in series of normals the corresponding value is $615 \mu\text{l}/30 \text{ min}/\text{gram}$.

Differences between normal glands both given inhibitors.

The percentage decrease in the series of bilaterally denervated glands was in the previous section found to be the same as that in the series of normal glands when one gland was made more sensitive than its fellow one by inhibitors. It was therefore thought to be of interest to compare the percentage decrease in enzymic activity when both glands were made supersensitive by inhibitors, one gland more sensitive than the other. This type of experiment was made on 4 cats. The results are shown in table VII. It can be seen that the percentage activity of the more sensitive gland in terms of the less sensitive, although this gland was also supersensitive, is of the same order as the figure in the comparable series of normals and bilaterally denervated glands. The mean value of the difference between the right and left gland, using figures for homogenate ACh was $187 \mu\text{l}/30 \text{ min}/\text{gram}$. The amounts given to induce increased sensitivity varied between 2 and $10 \mu\text{g}$ and the dose given to the other gland in addition between 4 and $10 \mu\text{g}$ of mintaköl.

Sensitivity to adrenaline and pilocarpine after inhibitors.

In several cases adrenaline was also tested, especially on submaxillary glands, before and after the injections of inhibitors. It was found that the sensitivity to this drug was not changed by the inhibitors in the amounts used here.

Pilocarpine was in several experiments used to induce secretion after the injection of the inhibitors. In this case comparison between response before and after inhibitors could obviously not be made. In the groups when a supersensitivity to cholinesters was induced on one side by inhibitors there was found no difference in the response to pilocarpine between the two sides and in the groups when the supersensitivity to cholinesters

caused by denervation was abolished by inhibitors, there was a much greater secretory response to pilocarpine from the denervated gland.

Discussion.

The intention of the present experiments was to get some information concerning the possible rôle played by changes in cholinesterase for the supersensitivity after denervation by using cholinesterase inhibitors. This could be done by comparing the decrease in enzymic activity caused by denervation with that after injection of inhibitors given so as to induce a supersensitivity. However, it could be that there are enzymes of different localization in the gland, enzymes in some places not being of importance for the destruction of injected drugs. This consideration made it desirable to use some different methods for preparing the enzyme sample to see whether this caused any difference in the results. In previous investigations on the decrease in cholinesterase activity after denervation, the supernatant of the homogenate (3,000 rev. p. m.) was used (STRÖMBLAD 1955 b and 1956). Since it was shown for submaxillary glands (KAHLSON and RENVALL 1956) that the supernatant contained some 30 per cent of the activity of the crude homogenate, it was decided to use the crude homogenate and the supernatant as enzyme samples.

It was confirmed in the present experiments that the supernatant contained some 30 per cent of the activity of the homogenate in normal submaxillaries and the same distribution was found for denervated glands. The supernatant of the homogenate of the parotid glands was found to contain some 80 per cent of the cholinesterase activity of the crude homogenate in normals, in pre- and postganglionically parasympathetically denervated glands, in sympathetically denervated glands and in glands from animals treated with an atropine-like substance. Thus there was in these experiments neither for submaxillary nor for parotid glands any indication of different types of cholinesterases. The significance, if any, of the difference between submaxillaries and parotids as to the distribution between enzyme in the homogenate and supernatant is unknown. For a discussion of soluble and particulate cholinesterases the reader is referred to SMALLMAN and WOLFE (1956). Anyhow, the difference between submaxillaries and parotids does not seem to be due to the fact that the sub-

maxillaries contain more enzyme and that the solubility in the supernatant is limited, since the changes in the supernatant generally reflected those in the homogenate and since the same results were found in experiments with a less dilution of the homogenate with buffer (STRÖMBLAD 1957).

Even if there were enzymes of different localization and function in the gland this would be of less importance for the main question of this work provided denervation and enzyme inhibitors affected the enzyme of the gland in the same fashion. Some evidence in favour of a similarity was found in the experiments described in the last sections; the denervated glands and glands injected with inhibitors needed the same further decrease in enzymic activity to yield a greater amount of saliva on injection of cholinesters. The percentage decrease was the same as in the comparable series of normals, but the difference in enzymic activity between the two glands constituting a pair was less in these series than in the series of normal glands.

From the experiments with inhibitors, it is concluded that a decrease by about 30 per cent in enzymic activity is necessary to affect the sensitivity to cholinesters both in submaxillaries and parotids.

The decrease in enzymic activity found after preganglionic parasympathetic denervation of submaxillaries and parotids was of the same order when the for the "enzyme-theory" most favourable figures *i. e.* total figures were used. Thus it is just possible that enzyme decrease may play a part in the supersensitivity caused by this type of denervation. On the other hand enzyme decrease cannot be the whole explanation, since the supersensitivity after preganglionic denervation is much more pronounced than that found after inhibition of enzymes to the level reached after this type of denervation. To get more evidence of this the normal submaxillary gland contralateral to a denervated submaxillary gland was injected with inhibitors until the sensitivity of the two glands were about equal. The enzymic activity was then considerably less in the gland given inhibitors than in the denervated gland.

After postganglionic parasympathetic denervation the decrease in enzymic activity towards ACh and MCh was greater than the decrease found when inhibitors caused a just detectable sensitization. The supersensitivity after this type of denervation could, however, not be wholly explained by lessened enzymic activity,

since in order to get a supersensitivity of the same degree by using inhibitors a greater fall in enzymic activity was necessary than that found after the denervation.

Could it be that the difference in supersensitivity reached after pre- and postganglionic denervation is due to the difference in enzymic activity? The experiments here presented seem to allow the conclusion that a great deal of the difference is due to this; other factors operating can, however, not be excluded.

It seems reasonable to assume that factors other than decrease in enzymic activity are at play in causing the supersensitivity after sympathetic denervation, since no changes in cholinesterase activity were found after this type of denervation.

In experiments when the effect on the sensitivity of enzyme inhibitors are compared with the effect of denervation, the question arises whether the inhibitors have some action on the sensitivity that is not shared by the procedure of denervation. There are two possibilities. The inhibitor can have an action of its own apart from inhibiting the intended enzyme. Such an action has been reported for Sarine and DFP on voluntary muscle (GROBLEWSKI, McNAMARA and WILLS 1956), and it could be that mintakol had a secretagogue effect. The second possibility is that the drug by inhibiting the enzyme can cause a local accumulation of acetylcholine; when using higher doses of this inhibitor a continuous flow of saliva is evoked (EMMELIN and STRÖMBLAD 1957). Both these possibilities would tend to increase the effect of injected secretory drugs, and the degree of inhibition found would therefore be too low. It is true that no secretion was evoked by the inhibitor alone in the present experiments, but the drug could act by lowering the threshold.

There are two observations in the present experiments that speak against the two sources of error under discussion. Firstly, mintakol and TEPP were found to be equal in their effects, though an action ascribable to organic phosphorous compounds generally is thereby not eliminated. Secondly, any concealed action of the inhibitors, as outlined above, would be evident also when adrenaline or pilocarpine were used to induce secretion. No potentiation of the secretory effect of these drugs was found with the amounts of inhibitors used here. The experiments with adrenaline and pilocarpine also show that in salivary glands, unlike what has been proposed for the iris (BURN and PHILPOT 1953), the supersensitivity to adrenaline is not caused via changes

in the activity of cholinesterases. Experiments bearing on this particular problem have earlier been described (STRÖMBLAD 1956) for salivary glands and the same conclusion as the present one was drawn.

Summary.

Experiments on the cholinesterase activity of homogenates of normal and denervated salivary glands are described.

The percentage decrease after various types of denervations was the same as found earlier (STRÖMBLAD 1955 b) when the supernatant of the homogenate was estimated.

The changes in enzymic activity after denervations were compared with those found when mintakol (or TEPP) was injected into the glands via the secretory duct in amounts causing supersensitivity.

It was inferred that the supersensitivity found after denervations could not be wholly due to a decrease in cholinesterase activity. In case of preganglionic denervation it is doubtful if enzyme decrease could play any part in the supersensitivity, while in case of postganglionic parasympathetic denervation it was very suggestive. Experiments are also described which are in accord with a suggestion previously proposed that the difference in sensitivity between a pre- and postganglionically denervated parotid gland is due to the greater fall in cholinesterase after postganglionic as compared with preganglionic denervation.

The supersensitivity caused by excision of the superior cervical ganglion could hardly be attributed to a decrease in cholinesterase activity of the gland.

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The Rectum-Liver Temperature Gradient in Man¹.

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In previous communications (GRAF, PORJÉ and ALLGOTH 1955, GRAF 1957) it has been reported that — against general belief — the human liver does not have the highest temperature in the body. Contrary to what might have been expected the rectal ampulla was found to have a temperature 0.2 to 0.6°C respectively $0.43 \pm 0.10^{\circ}\text{C}$ higher than the liver parenchyma.

These observations are essentially in accordance with the findings of EICHNA, BERGER, RADER and BECKER (1953), who found that with few exceptions the rectal temperature in man was higher than in the hepatic veins. They are, however, contradictory to several reports based on animal experiments, from the classical data of CLAUDE BERNARD (1854) to more recent results (LEFÈVRE 1911, NEDZEL 1934, MARSHAK 1939, DIETRICK and FRITTS 1952). On the other hand, SOLLBERGER (1957) has recently found that in chickens the rectal temperature was $0.15 \pm 0.02^{\circ}\text{C}$ higher than the temperature of the liver.

Method.

Copper-constantan thermocouples were used, the junctions measuring 1.0 mm in length and the wires having a diameter of 0.2 mm. The wires intended for the different organs were equally long and insulated in a polyethylene tube (the outer diameter of which amounted to 1.24 mm). One thermocouple was introduced into the

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The authors are indebted to Miss A.-M. ALLGOTH for skilful technical assistance.

liver parenchyma immediately following a liver biopsy, the junction and its wire left inserted in the liver 8–12 cm. The technique has been described previously (GRAF, PORJÉ and ALLGOTH 1955). The second thermocouple was introduced into the rectal ampulla just above the sphincter.

The wires were connected with a galvanometer. Two galvanometers of different sensitivity were used in different experiments, the deflections of which were recorded by means of a "Nachlaufschreiber" (Fa. Dr. B. Lange, Berlin). The more sensitive instrument yielded a deflection of 20 mm per 0.1° C difference, the less sensitive one of 5 mm per 0.1° C difference.

Apart from these two thermocouples a separate junction was introduced in the stomach and another used for the measuring of skin temperature. Gastric and skin (manubrium) temperatures were thus measured with the aid of a common reference junction, kept in room temperature but built to compensate for variations of the latter (manufactured by Ellab, Copenhagen). It did not include continuous recording, and thus successive values had to be read from the galvanometer scale at short intervals. Checking of this apparatus was performed in a constant temperature water-bath and its error found to be close to $\pm 0.04^{\circ}$ C.

Material.

Nine male patients and one female, aged 35 to 61 years, were used for this study, all being admitted to the hospital on account of an alleged liver injury. The biopsy specimens revealed that three patients had a slight to moderate fatty infiltration of the liver, in the other cases no pathological findings whatsoever of the hepatic structures were found. Laboratory findings did not suggest any functional failure of the liver.

Results.

The gradient was first observed during a control period of 30 to 60 minutes. The mean value of the rectum-liver gradient from the ten cases was found to be $+0.18 \pm 0.03^{\circ}$ C ($\sigma = 0.095$), in all cases the rectal temperature was higher than the liver temperature throughout the "spontaneous" period (range 0.08 to 0.34° C). After this control period the influence of different agents on the gradient and on the gastric and skin temperatures was studied. These agents could be divided in two groups: those increasing the rectum-liver gradient and those acting in the opposite direction. In a previous study (GRAF 1957) it was found that when 0.001 g histamine (dihydrochloride) was administered subcutaneously a skin temperature rise ensued with a simultaneous fall in temperature in the stomach, liver and rectum.

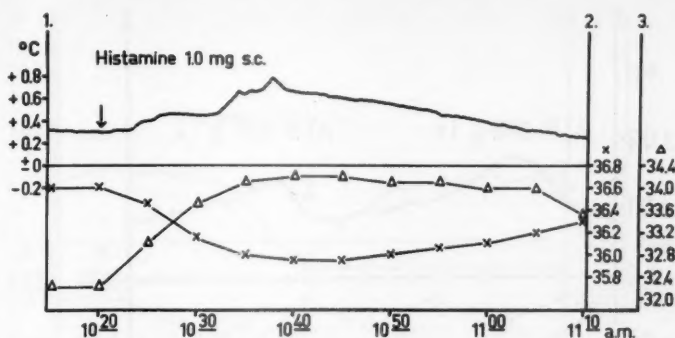


Fig. 1. Effect of 1.0 mg histamine subcutaneously. Abscissa: A base line has been drawn corresponding to the ± 0 level of the rectum/liver gradient. Time indicated at the bottom. Ordinate: 1. the gradient (drawn as an uninterrupted line), position above base line means a higher rectal than liver temperature. 2. gastric temperature (\times — \times) and 3. skin temperature (Δ — Δ) in $^{\circ}\text{C}$, the respective symbols indicated (different scales).

The fall in temperature measured in the rectum was delayed and less pronounced than in the stomach and the liver.

When applying the method outlined above it was found that concomitant to the stomach temperature fall, an increase of the rectum-liver gradient occurred. Fig. 1 shows one case, where before the injection of 0.001 g histamine the rectum-liver gradient was 0.33°C and 18 minutes afterwards it had increased to 0.78°C . The maximum of the gradient increase corresponded closely in time to the lowest temperature in the stomach and to the skin temperature peak. The gradual decrease of the rectum-liver gradient towards its original level also corresponded to the return of the gastric temperature versus its pre-injection value. In two further cases the corresponding maximal change of the rectum-liver gradient was from $+0.10$ to $+0.27^{\circ}\text{C}$ at 20 minutes and from $+0.18$ to $+0.37^{\circ}\text{C}$ at 12 minutes. In these cases, too, the inversed parallelism between the gradient and the absolute gastric temperature was very conspicuous.

Fig. 2 shows the effect of adenosinetriphosphoric acid (ATP) when injected intravenously in doses of 0.01 g. Immediately following each injection an increase of the gradient occurred, whereas the gastric temperature fell and the skin temperature rose after the first but not after the second injection. The lack of effect of the second injection on the last-mentioned tempera-

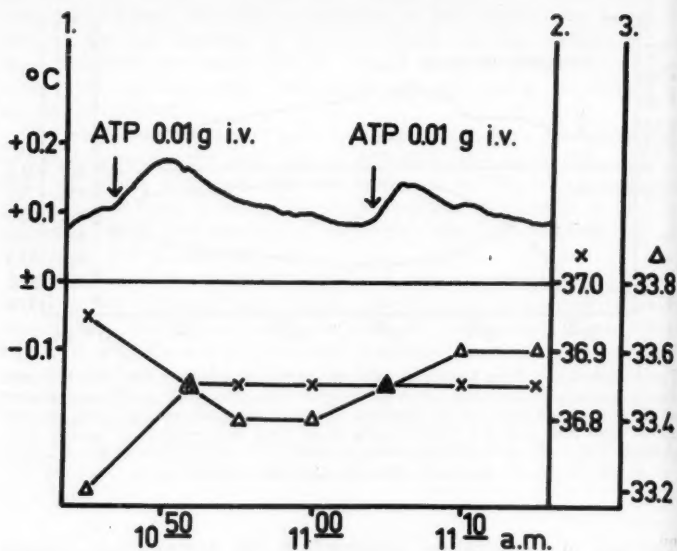


Fig. 2. Effect of adenosine triphosphoric acid (given twice, 0.01 g i.v.). Symbols as in fig. 1.

tures may be explained by the lesser sensitivity of the equipment intended for measurement of the same.

Among agents further found to increase the rectum-liver gradient was Decholin (10 ml of a 20 % solution given intravenously), though giving an unambiguous effect only in one out of two cases. In one subject the injection of Decholin elicited no appreciable effect on the gradient but a slight gastric temperature fall and a moderate skin temperature drop. In the second patient the rectum-liver gradient increased, the gastric temperature fell and the skin temperature rose (for interpretation see below).

The gradient also displayed an immediate increase when the subjects ingested some 200 ml water of 3 to 7° C. This experiment was not performed in order to study quantitatively the cooling of the liver by ingestion of cold water but merely as an extra means to check the direction of the gradient.

The decreasing effect of some pharmacological stimuli on the rectum-liver gradient was established, namely that of pitressin, epinephrine, norepinephrine and of artificial fever.

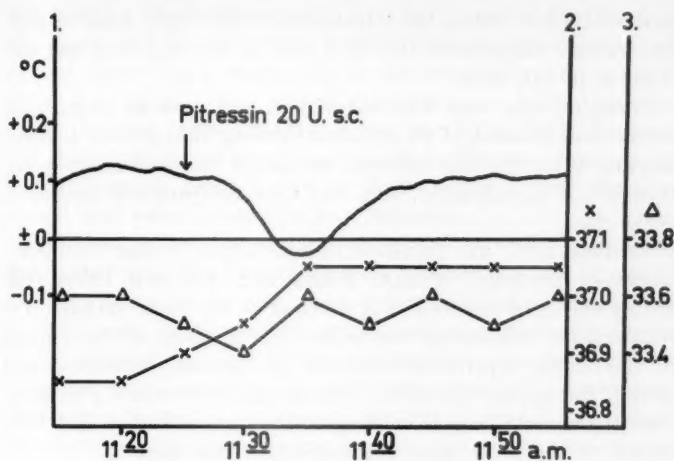


Fig. 3. Effect of pitressin (20 Units).

Symbols as in figs 1 & 2.

A reversal of the gradient occurs during five minutes.

Pitressin (20 I.U. subcutaneously) given in two cases elicited a uniform decrease of the gradient simultaneously with the gastric temperature rise and skin temperature drop. Fig. 3 shows one case where the rectum-liver gradient decreased from $+0.11$ to -0.025°C and was thus reversed. The maximum reaction occurred at 10 minutes and had passed at 20 minutes, when the gastric temperature had risen 0.25°C and reached a plateau. The skin temperature drop in this case was fairly transient. In the second case pitressin reversed the gradient from $+0.09$ to -0.06°C . In this case the gastric temperature continued to rise even after the gradient had returned to its original value.

The effect of epinephrine was tried in two cases. A solution of $10\text{ }\mu\text{g}$ per ml was administered intravenously with the rate of $1\text{--}2\text{ ml/minute}$. In both patients pallor developed as well as polypnoea and the subjective sensation of oppression. A decrease of the rectum-liver gradient ensued in both cases, from $+0.02$ to -0.11°C and from $+0.10$ to $+0.02^{\circ}\text{C}$ respectively. The decrease of the gradient appeared immediately when the infusion had started and was followed by a return to or increase above the original value within 5–10 minutes after the infusion had stopped. In both cases given epinephrine the skin temperature

was unchanged during the infusion but rose steeply after its end. The gastric temperature fell by 0.2°C in one case but was unchanged in the other.

Norepinephrine was administered in one case as continuous intravenous infusion of $25\text{ }\mu\text{g/minute}$ during 10 minutes. A slight decrease of the gradient followed, the gastric temperature dropped, though not significantly, and the skin temperature showed a slight rise.

Artificial fever was provoked in one subject. It has been demonstrated previously (GRAF, PORJÉ and ALLGOTH 1955) that during the temperature rise a reversal of the usual rectum-liver temperature relationship can occur. The gradient of the patient in this single experiment followed the pattern mentioned and already during the early phase of temperature elevation a crossing-over of the rectal and liver temperatures was recorded. The later phases of the fever were not studied in this case.

Discussion.

Apart from the observations quoted above by GRAF, PORJÉ and ALLGOTH (1955) and GRAF (1957) the temperature of the human liver has not previously been directly measured. It has been anticipated, though, on the basis of CLAUDE BERNARD's (1854) observations on animals and further work in the same genre, that the human liver ought to be the main heat-producing centre in the human body and consequently have a higher temperature level than any other organ. Certain reports have, however, yielded indirect evidence against this prevailing conception. IPSEN (1926) measured the temperature of the peritoneal cavity and found that the pelvis often displayed a higher temperature level than the upper part immediately below the liver. It could further be referred to the work of EICHNA, BERGER, RADER and BECKER (1953) quoted above.

In the present material of ten subjects the rectum-liver temperature difference was smaller than in the previous reports (GRAF, PORJÉ and ALLGOTH 1955, GRAF 1957). This must largely be due to the different measuring techniques employed but the fact that the rectal temperature exceeds that of the liver still holds true. This finding does not contradict the possibility that the liver's contribution to the body's heat production is high in comparison to other tissues: the high hepatic blood flow implies

that even a small temperature gradient between the blood of the portal and hepatic veins means a considerable contribution of heat. According to some authors the temperature of the portal blood is — judged from animal experiments — lower than that of the hepatic veins (JITARIU, KOCH and OTTO 1941, FEDOROV and SHUR 1942) but according to others (MILLER, SCHASTNAYA and JUTKEVICH 1940) higher — if the latter is true the liver should still have a negative heat balance.

Constituting only a minor part of the structures supplying the portal vein the rectum and its temperature are of course not representative for the whole splanchnic system drained by the same vein. If the majority of the organs and tissues belonging to the alimentary canal have a temperature considerably lower than the rectal ampulla, it could well result in a lower temperature in the portal vein than in the liver. Judged from available literature temperature measurements in the portal vein of man have, however, not been carried out.

Administration of histamine, ATP and Decholin increased the rectum-liver gradient. Concerning histamine this finding confirms previous data-(GRAF 1957). When histamine causes a dilatation of skin blood vessels the heat loss from the body surface increases. The cooled blood recirculating from the skin will lower the temperature of the inner organs proportional to their rate of blood flow. Thus it could be expected that the liver temperature drop would be greater than that of the rectum. Besides, it has been demonstrated, that histamine causes an increase of the liver blood flow (BRADLEY, INGELFINGER and BRADLEY 1952, GRAF 1957, GRAF, GRAF, ROSELL and ALLGOTH 1957).

Concluded from studies on liver blood flow in man by means of measuring the thermal conductivity of the liver (GRAF, GRAF, ROSELL and ALLGOTH 1957) ATP increases liver blood flow considerably. According to NEUMAYR (1956) Decholin has a similar effect. It is reasonable to assume that this is largely the cause of the gradient increase elicited with the said substances as reported above. Agents known to elicit a decreased liver blood flow were also found to decrease or even reverse the gradient. On studying the behaviour of stomach, liver and rectum temperatures under influence of pitressin GRAF (1957) has found (data not yet published) a temperature increase appearing in all organs and simultaneous with a skin temperature drop. The temperature increase in the inner organs was of the order

stomach > liver > rectum (the difference between the induced change of rectal and liver temperatures had a significance of $P < 0.05$). This finding corresponds exactly to the decreased gradient reported above to occur after the administration of pitressin. If the rise in temperatures of all inner organs measured is secondary to the lessened heat loss from the body surface — it is assumed then, that pitressin does not exert any significant influence on the local heat production in the organs in question — the mutual differences in temperature rise between the organs may be due to the fact that the changes in blood flow induced with pitressin are not equally pronounced in the various organs. In a study of the effect of pitressin on the liver blood flow (GRAF, GRAF, ROSELL and ALLGOTH 1957) it has been shown that a sharp diminution occurs immediately. Provided that the blood flow decrease in the rectal wall is less pronounced the shift in temperature relationship (reversal of the gradient) is thus easily understood.

The actions of epinephrine and norepinephrine are less univocal. The first-mentioned substance caused an initial decrease (during the infusion) and a subsequent increase (after the infusion) of the rectum-liver gradient. This behaviour corresponds to a tracing of the liver blood flow by means of thermoelectrical methods (NEUMAYR 1956, GRAF, GRAF, ROSELL and ALLGOTH 1957). On the other hand, liver blood flow estimation with bromsulphthalein has not supplied evidence of a diphasic action but merely of an increased flow (BEARN, BILLING and SHERLOCK 1951, BRADLEY, INGELFINGER and BRADLEY 1952). Animal experiments suggest that the effect of epinephrine on liver blood flow is of a very complex character (McMICHAEL 1932, BAUER, DALE, POULSSON and RICHARDS 1932, GRAYSON and JOHNSON 1953). The interpretation of its action on the rectum-liver temperature gradient also seems to require further work. CRILE and ROWLAND (1922) stated that in the rabbit the temperature of liver and muscle did not change under the influence of a single dose of epinephrine whereas that of brain and thyroid rose considerably. For muscle tissue of the dog this observation was not confirmed by CASKEY and SPENCER (1925) and might thus be open to question also concerning the human liver. Concerning norepinephrine the gradient decrease recorded in the present investigation is in complete accordance with the blood flow decrease obtained with the same agent by means of recording the thermal

conductivity of the liver (GRAYSON and JOHNSON 1953, GRAF, GRAF, ROSELL and ALLGOTH 1957). It should be mentioned that with bromsulphthalein technique applied on the dog, the corresponding effect has not been obtained (SMYTHE, GILMORE and HANDFORD 1954).

The effects of epinephrine and norepinephrine in the present work were not nearly so pronounced as that seen after pitressin administration. This is evidently a matter of dosage and related to the relative lack of side-actions with pitressin as compared to epinephrine and norepinephrine, necessitating a more careful attitude and more modest dosage of the two last-mentioned agents when applied to human beings.

One single experiment with artificial fever included in the series reported is not possible to comment otherwise than that the reversal of the gradient provides further evidence that the occurrence of a temperature excess in the liver is inherent in the initial phase of the fever. This increased heat production must be considerable as it has been stated that a pronounced increase of the hepatic blood flow occurs during the pyrogenic reaction (BRADLEY and CONAN 1947). The heat production in the liver must compensate for the increased heat convection with the blood stream. The reversal of the gradient also establishes that the elevated liver temperature cannot be due to mere decrease of heat loss from the body surface. In this case the different organ temperatures should have reacted uniformly.

Summary.

1. Copper-constantan thermocouples were introduced, one into the human liver, the other in the rectal ampulla. The temperature gradient between the rectum and the liver was recorded continuously with a calibrated measuring system.
2. In all ten cases examined the rectal temperature was higher than that of the liver. From experiments under basal conditions a mean rectum-liver temperature gradient was obtained of $+0.18 \pm 0.03^{\circ} \text{C}$.
3. The effect of various pharmacological agents on the gradient was studied. Substances known to increase the liver blood flow were found to increase the gradient and vice versa. A reversal of the gradient occurred during artificial temperature elevation (initial phase of fever).

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The Albumin/Globulin Ratio in Plasma and Exudate of Chicks Suffering from Exudative Diathesis.

By

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The cause of the exudation of plasma occurring in chicks reared on vitamin E deficient diets containing polyenoic fatty acids has been sought in capillary damage (DAM and GLAVIND 1939, 1940), and also in a lowered albumin/globulin ratio (GOLDSTEIN and SCOTT 1956).

DAM and GLAVIND (1939) found a protein content of the same order of magnitude in exudate and plasma, thereby characterizing the former fluid as an "exudate" as distinguished from a transudate (the low protein fluid of most edemas). They found lower values for the protein content of plasma from "exudate chicks" than from plasma of chicks on a normal diet. In the two instances where the ratio globulin/total protein was determined they found no difference between plasma and exudate in this respect. DAM and GLAVIND (1940) showed that intravenously injected trypane blue passed out into the muscles of exudate chicks to a larger extent than in chicks receiving d,l- α -tocopherol acetate. DAM (1944) found no significant difference in specific gravity between serum from fasting chicks with exudates (in the resorption stage) and serum from fasting chicks on a commercial chicken diet. Therefrom it was concluded that capillary damage was the major cause of the exudation.

Table 1.

Commercial chicken ration¹.

(Starting and growing mash, manufacturer: "Selandia", Copenhagen.)

"Pantoribin" ³	3
Dried buttermilk	4
Meat and bone meal	10
Fish meal	7
Soybean oil meal (defatted ground soybeans)	6
Ground yellow corn	10
Ground wheat	20
Ground barley	19
Ground oats	10
Wheat middlings	8.67
"Selamin" salt mixture ³	2
"Deltamin A-D, strong" ⁴	0.33
	100.00

¹ Containing 16 % digestible protein, and 600 i.u. vitamin A, 300 i.u. vitamin D₃, 0.1 mg thiamin, 0.3 mg riboflavin, 1.05 g nicotinic acid amide, 0.51 mg pantothenic acid per 100 g.

² Wheat germ 90 parts, wheat middlings fortified with vitamin D₃, thiamin, riboflavin, nicotinic acid amide and pantothenic acid, 10 parts.

³ Manganous sulfate	0.0792
Cobaltous sulfate	0.0024
Ferrous sulfate	0.1440
Cupric sulfate	0.0072
Zink sulfate	0.0048
Magnesium sulfate	0.0024
Dicalcium phosphate	0.4000
Calcium carbonate	0.9600
Sodium chloride	0.4000
	2.0000

⁴ Wheat middlings fortified with vitamins A and D₃.

GOLDSTEIN and SCOTT (1956) carried out paper electrophoresis on exudate and plasma of exudate chicks receiving a diet with torula yeast as the source of protein, and compared the results with those obtained with plasma of chicks on a similar diet plus d- α -tocopheryl acetate or on a normal diet. They found a decrease in total plasma protein and more markedly in the albumin of chicks suffering from exudative diathesis. The exudates produced electrophoretic patterns which were qualitatively similar to those of the plasmas from the same chicks. Further, a higher albumin/globulin ratio was found in the plasma of chicks on a stock ration than in chicks on the basal diet supplemented with vitamin E.

CREECH, FELDMAN, FERGUSON, REID and COUCH (1957) in studies with turkeys found that under certain circumstances vitamin E as well as brewer's yeast could increase the albumin/globulin ratio of serum as determined by paper electrophoresis.

Table 2.
Artificial diet no. 1800.
(Vitamin E deficient.)

Torula yeast 3N ¹	58.50 g
Salt mixture ²	5.17 g
Vitamin mixture ³	0.10 g
Choline chloride	0.20 g
Glucose ⁴	36.03 g
	100.00 g

This amount of diet was supplemented with 1 mg dicalcium salt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester (Synkavit "Roche"). Vitamins A and D₂ were given as a solution⁴, 0.1 ml twice a week per animal. This corresponds to 250 i.u. A and 20 i.u. D₂ per day.

¹ From Lake States Yeast Corporation, Rhinelander, Wisconsin.

² DAM and SØNDERGAARD (1953).

³ "Chemically pure". From A/S Fiducia, Copenhagen.

⁴ Made up from crystalline vitamin A acetate ("Roche") 1 g; crystalline vitamin D₂ ("Roche") 0.0058 g; ethyl alcohol 100 ml; "Tween 80" 64 g; and distilled water to make a total volume of 330 ml.

In order to obtain some further information on the changes in proteins occurring in chicks with exudative diathesis we have carried out Tiselius electrophoresis studies on plasma and exudate from chicks on a vitamin E-free torula yeast diet resembling that used by GOLDSTEIN and SCOTT (1956) and SCOTT, HILL, NORRIS, DOBSON and NELSON (1955), as well as on plasma of chicks receiving the same diet supplemented with 10 mg % d,l- α -tocopherol acetate and on plasma from chicks on a normal commercial diet. Further, we have determined the mobility of various of the plasma fractions.

The chicks (cross-breed of Light Sussex and White Leghorn) were obtained day-old from the dealer and fed the commercial diet indicated in Table 1. After 8 days a certain number of them were shifted to the artificial diet no. 1800, indicated in Table 2, with or without 10 mg % d,l- α -tocopherol acetate (Ephynal, "Roche").

Blood and exudate fluid were taken out by means of a syringe containing 0.5 ml of a 0.1 molar sodium oxalate solution. In the case of blood, 4 ml was taken from the jugular vein. Exudate fluid, 2.5 to 4 ml, was taken from chicks with large fresh subcutaneous exudates.

The samples of plasma and exudate were placed in bags of Visking tubing and dialyzed for 48 to 72 hours at 5° C against a buffer of 0.015 M diethylbarbituric acid, and 0.100 M sodium-diethylbarbiturate at pH 8.6. This solution was changed every 24 hours. After dialysis the samples were centrifuged and diluted to the three- or four-fold volume. The conductivity was measured by means of a Leeds & Northrup Co. Wheatstone Bridge assembly, with conductance cell mounted in Dewar vessels with finely ground ice at 0° C.

Table 3.
Albumin/globulin ratio.

Chick no.	Sex	Days on		Normal diet		Artificial diet plus vitamin E		Artificial diet without vitamin E			
		normal diet	artificial diet	Plasma		Plasma		Plasma		Exudate	
				desc.	asc.	desc.	asc.	desc.	asc.	desc.	asc.
5873	m	44	0	0.70	0.50						
5862	f	46	0	0.79	0.75						
5863	f	47	0	0.80	0.78						
5864	m	48	0	0.99	0.91						
6171	m	29	0	0.68	0.75						
6172	m	29	0	^a 0.74	^a 0.76						
6173	m	29	0	0.61	0.63						
5813	m	8	18			^a 0.65	^a 0.53				
5815	f	8	19			0.65	0.64				
5817	m	8	20			0.62	0.67				
5812	m	8	22			0.65	0.70				
5820	m	8	32			0.59	0.44				
5796	f	8	18					^a 0.26	^a 0.27	0.90	0.60
5807	m	8	19					0.39	0.25	0.75	0.75
5801	m	8	20					0.39	0.39	0.92	0.72
5800	m	8	21					0.25	0.25	0.55	0.55
5797	m	8	22					0.30	0.30	^a 0.60	^a 0.55
Average				0.76	0.72	0.63	0.60	0.32	0.29	0.58	0.62
Average of desc. and asc.				0.74		0.62		0.31		0.60	

¹ This chick received a supplement of 0.1 % orotic acid in the diet.

² Pattern shown in Fig. 2.

³ " " " " 3.

⁴ " " " " 4.

⁵ " " " " 5.

The electrophoresis was carried out in a Perkin-Elmer portable Tiselius Electrophoresis Apparatus in a cell of 2 ml capacity. The duration of the electrophoresis varied from 5,000 to 7,000 seconds. The patterns obtained were, after enlargement, measured with a planimeter, and the percentage composition of albumin and globulin calculated from the respective areas (MOORE, 1949). The base line used in the calculation was determined by measuring the top and the bottom edges and taking the average.

Both the descending and the ascending sides were used. The mobilities of the different proteins were made from measurements of the descending sides only.

Fig.
x
+
O
●
Ab

Fig. 2

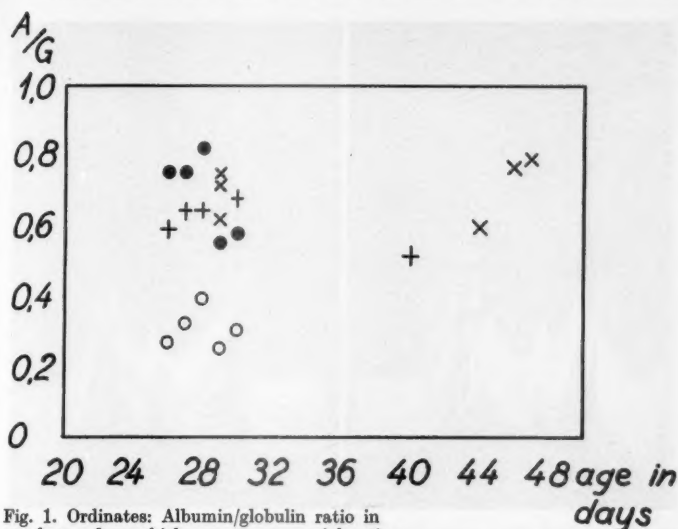


Fig. 1. Ordinates: Albumin/globulin ratio in
 × plasma from chicks on a commercial ration,
 + plasma from chicks on an artificial torula yeast diet plus vitamin E,
 o plasma from chicks on an artificial torula yeast diet without vitamin E,
 ● exudate from chicks on an artificial torula yeast diet without vitamin E.
 Abscissae: Age in days.

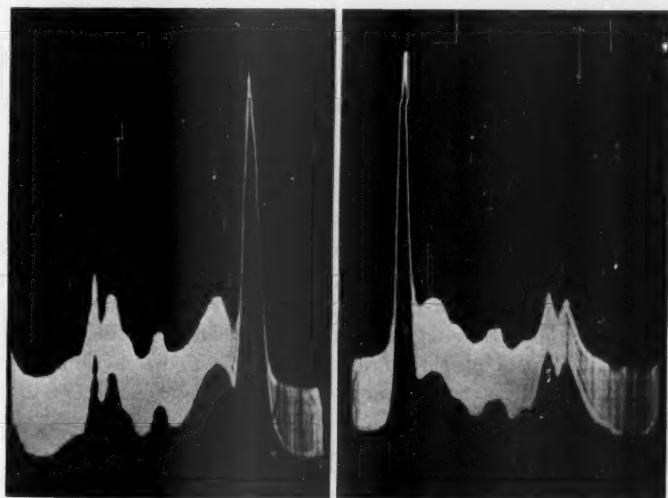


Fig. 2. Electrophoretic pattern of plasma from a chick (no. 6172) fed a normal commercial diet for 29 days.
 Left: descending, right: ascending part.

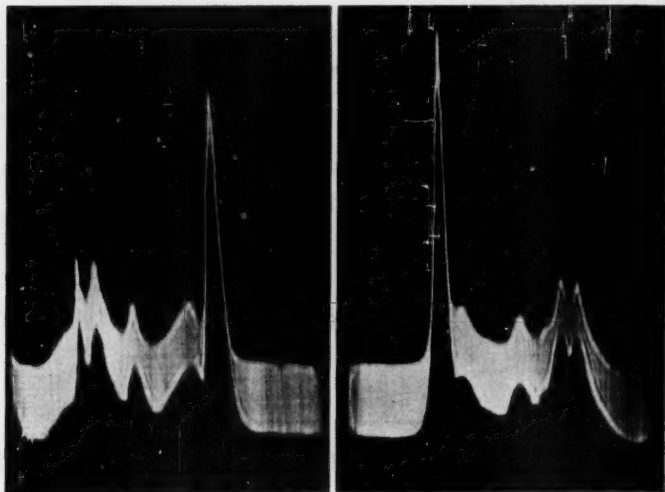


Fig. 3. Electrophoretic pattern of plasma from a chick (no. 5813) fed a commercial diet for 8 days and thereafter an artificial diet with torula yeast and vitamin E for 18 days.

Left: descending, right: ascending part.

Results and Discussion.

The albumin/globulin ratios are presented in Table 3 and Fig. 1.

Figs. 2—5 show typical electrophoretic patterns for the three different plasmas and the exudate.

The mobilities of the different plasma proteins are shown in Table 4.

The albumin-globulin ratios were found to be largely the same for plasma of chicks on the normal diet, on the diet with torula yeast supplemented with vitamin E, and for the exudate. Lower albumin/globulin ratios were found only for plasma of chicks on the vitamin E-deficient torula yeast diet.

The mobilities of the various plasma protein fractions were not influenced by the dietary regimen. The protein fractions in the exudate had the same mobilities as the corresponding fractions in the plasmas.

It is yet undecided whether the lowering of the albumin/globulin ratio in the plasma of the exudate chicks is caused by the exudation or is present before the exudation occurs. In the

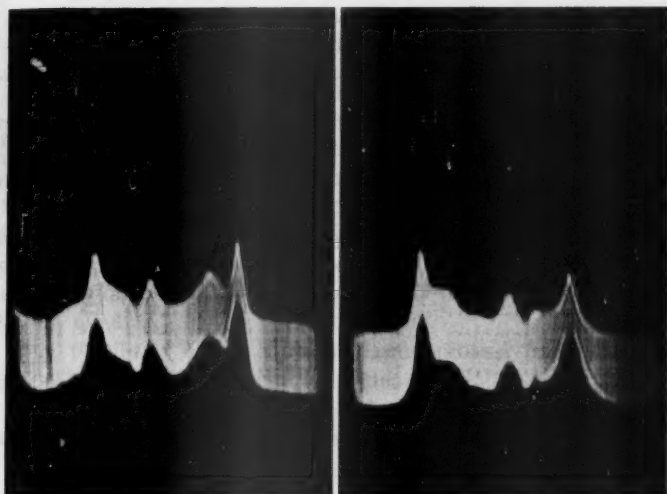


Fig. 4. Electrophoretic pattern of plasma from a chick (no. 5796) fed a commercial diet for 8 days and thereafter an artificial diet with torula yeast without vitamin E for 18 days. The chick had a large subcutaneous exudate.
Left: descending, right: ascending part.

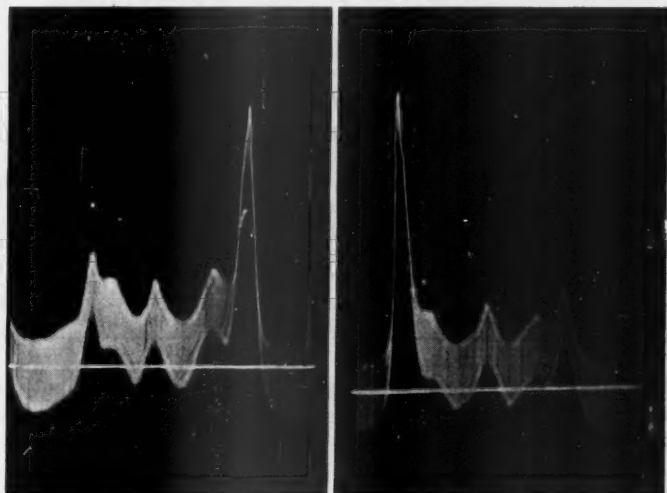


Fig. 5. Electrophoretic pattern of fresh exudate from a chick (no. 5797) fed a commercial diet for 8 days and thereafter an artificial diet with torula yeast without vitamin E.
Left: descending, right: ascending part.

Table 4.

Mobilities $\times 10^{-5}$ cm² volt⁻¹ sec⁻¹ measured on the descending side.

Fraction number:			1	2	3	4	5
Common designation:			Albu- min	α_1 Globu- lin	α_2 Globu- lin	β Globu- lin	$\gamma + \theta$ Globu- lin
Chick no.	Diet						
5873	Normal	Plasma	6.3	5.7	3.7	2.4	2.1
5862	"	"	6.5	5.8	3.8	2.3	1.9
5863	"	"	6.2	5.4	3.4	2.4	2.0
5864	"	"	6.6	6.0	3.9	2.6	2.2
6171	"	"	6.4	5.6	3.8	2.4	2.1
6172	"	"	6.4	5.6	3.8	2.4	2.1
6173	"	"	6.4	5.6	3.8	2.4	2.1
		Average	6.4	5.7	3.8	2.4	2.1
5813	Artificial plus vi- tamin E	Plasma	6.9	5.9	4.0	2.6	2.2
5815	— " —	"	6.6	5.7	3.9	2.4	2.1
5817	— " —	"	6.4	5.6	3.8	2.4	2.1
5812	— " —	"	6.5	5.7	3.8	2.4	2.1
5820	— " —	"	6.7	5.7	3.8	2.5	2.1
		Average	6.6	5.7	3.9	2.5	2.1
5796	Artificial without vitamin E	Plasma	6.6	5.7	3.8	2.8	2.1
5807	— " —	"	6.3	5.5	3.5	2.4	1.9
5801	— " —	"	6.5	5.8	3.8	2.5	2.2
5800	— " —	"	6.5	5.9	3.9	2.6	2.2
5797	— " —	"	6.7	5.8	3.8	2.7	2.2
		Average	6.5	5.7	3.8	2.6	2.1
5796	Artificial without vitamin E	Exudate	6.3	5.9	4.1	2.8	2.4
5807	— " —	"	6.5	5.8	3.7	2.8	2.0
5801	— " —	"	6.4	5.5	3.9	2.8	2.1
5800	— " —	"	6.5	5.8	3.8	2.9	—
5797	— " —	"	6.8	5.7	3.8	2.9	2.1
		Average	6.5	5.7	3.9	2.8	2.1

latter case the lower albumin/globulin ratio may contribute to the formation of exudate. This question will be subjected to further investigation.

The fact that the initial stage of the exudation is characterized by hemorrhage makes it seem likely that capillary damage is the most important factor in the initiation of the exudation process.

Summary.

Tiselius-electrophoresis was carried out on plasma from chicks on a normal commercial chicken ration, on a torula yeast containing artificial diet with or without vitamin E, and on exudate from chicks on the last mentioned diet.

The albumin/globulin ratios were found to be largely the same for plasma of chicks on the normal diet, on the diet with torula yeast supplemented with vitamin E, and for the exudate. Lower albumin/globulin ratios were found only for plasma of chicks having exudate.

The mobilities of the various plasma protein fractions were not influenced by the dietary regimen.

The protein fractions in the exudate had the same mobilities as the corresponding fractions in the plasmas.

Acknowledgements.

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Turnover Rate of Unesterified Fatty Acids in Human Plasma.

By

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The discovery that heparin-induced clearing of lipemia is of lipolytic nature (SHORE, NICHOLS, and FREEMAN 1953, BROWN, BOYLE and ANFINSEN 1953) resulted in the elaboration of simple methods for determination of unesterified fatty acids (UFA) in the plasma (GROSSMAN, STADLER, CUSHING and PALM 1955, GORDON and CHERKES 1956, DOLE 1956, a) and led to an intense investigation of their variation under various circumstances. It is of special interest that increases in the concentration of UFA in plasma have been observed in a number of conditions associated with an increased transport of fat from the fat depots such as after a varying period of fasting (DOLE 1956, GORDON and CHERKES 1956), diabetic acidosis (LAURELL 1956) and carbon tetrachloride poisoning (SPITZER and MILLER 1956). These observations gave rise to the hypothesis that UFA constitute an important form of transport of fat in the plasma. Even in the highest concentrations observed ($3 \text{ mEq/l} = 80 \text{ mg/100 ml}$) UFA represent only a minute portion of the plasma lipids. Therefore if this fraction is important in lipid transport, its turnover rate must be high.

The purpose of the present investigation was to assess the turnover rate of UFA in human plasma. While the investigation was in progress, an article appeared by HAVEL and FREDRICKSON (1956), who showed a half life of 2 minutes for unesterified palmitic acid in plasma in a dog fasted for 20 hours.

In order to determine whether the increase in UFA during fasting and their decrease after the administration of glucose is reflected by their rates of turnover, experiments were carried out after varying periods of fasting and after the administration of glucose.

Experimental. The experiments were carried out on apparently healthy volunteers of both sexes, aged 20—35 years. They were divided into 3 groups. In one group the disappearance rate of intravenously injected labelled fatty acids was determined after 12—15 hours' fasting, in another group after 38—68 hours' fasting, and in a third 2 hours after administration of about 1 g of glucose per kg body weight.

Preparation of injection solution. Palmitic acid 1-C¹⁴ (spec. activity 477,000 c/min./mg—0.59 μ C/mg) and oleic acid 1-C¹⁴ (spec. activity 487,000 c/min./mg—0.60 μ C/mg) were courteously supplied by Professor S. BERGSTRÖM and stored dissolved in toluene. For every experiment an aliquot of the solution corresponding to 4.47 mg palmitic acid (2.6 μ C) or 4.02 mg of oleic acid (2.4 μ C) was evaporated in a stream of nitrogen in small test tubes and then neutralised with 0.02 N NaOH in slight excess at 75° C for palmitic acid and at 40° C for oleic acid. To the opalescent soap solution 3 ml of sterile 20 per cent human albumin solution (Kabi, Stockholm) and 3 ml saline were added. The solution obtained was then water clear (analysis of UFA in the albumin preparation used was 5.2 mEq/l corresponding to 1.8 mol fatty acids per mol albumin).

Sampling. After samples had been collected for quantitative determination of UFA, the solution prepared in the above mentioned way was injected for about 5 seconds into a cubital vein. (The injection needle and the tube with the injection solution were rinsed with water and ethanol and the washings were diluted to 10 ml. In a portion of this solution the amount of labelled fatty acids not injected was determined and from this the amount injected was calculated.)

4—5 ml blood was collected from the cubital vein in the other arm of the subject at intervals of 2—5 minutes. The blood was allowed to run directly into ice-cooled centrifuge tubes containing an anti-coagulant (EDTA-Na₂) and centrifuged in the cold.

One ml of the plasma obtained was extracted with 25 ml chloroform-methanol 2 : 1. 20 ml of the filtered extract (corresponding to 0.8 ml of plasma) was purified according to SPERRY and BRAND's (1955) procedure 1 after addition of 0.1 ml of concentrated acetic acid. This purification did not involve any loss of palmitic or oleic acid, as judged by experiments with addition of labelled acids. The samples were not studied for any loss of mono- or diglycerides.

Separation of UFA from the extract. In one experiment with labelled palmitic acid and one with labelled oleic acid the cholesterol esters, glycerides, free fatty acids, and phosphatides were separated chromato-

graphically according to BORGSTRÖM (1952). At most a total of 1.5 per cent of the activity added (corrected for dilution in the predicted plasma volume) was recovered in fractions other than the UFA.

It was therefore considered justified to use a simplified method for the preparation of the UFA. It is known that the extraction of free fatty acids from a lipid mixture in petroleum ether with an alkaline ethanol results in considerable hydrolysis, above all of the phospholipids and to the extraction of fatty acids liberated from the phospholipids to the alcoholic phase.

Since little or none of the labelled fatty acids can be recovered in esterified form, in the present experiments this hydrolysis is of importance only because the effect of dilution makes measurements of the radioactivity somewhat more difficult. It appeared probable that this hydrolysis might be diminished by replacing the commonly used strong alkaline unbuffered ethanol by buffered ethanol of lower pH.

In order to investigate the effect of different pH levels on the degree of hydrolysis, 4 ml of petroleum ether containing total lipids from 2 ml serum were extracted in glass-stoppered centrifuge tubes with 8 ml of a solution containing equal volumes of ethanol and M/15 glycine buffers of varying pH (pH 10, 11, 12 and 13). After removing the petroleum ether with a syringe the alcoholic phase was extracted twice with 2 ml of petroleum ether. After acidification to pH 3 with a predetermined amount of 0.4 N HCl the free fatty acids were extracted three times with 3 ml of petroleum ether and titrated with 0.02 N NaOH (Agla micrometer syringe, thymol blue as an indicator) after evaporation and addition of 3 ml of ethanol. For comparison, double determinations were also made of UFA from the same serum with the technique of GROSSMAN et al. 1955.

In order to assess the recovery of the fatty acids present, petroleum ether solutions of labelled palmitic and oleic acid were extracted in the same way.

For every test use was made of 0.2 mg palmitic acid 1-C^{14} (spec. activity 5,230 c/min./mg) and 0.25 mg oleic acid 1-C^{14} (spec. activity 3,610 c/min./mg), respectively. All the material extracted from the alkaline and from the acidified ethanol, respectively, was plated and the radioactivity was determined. The results are given in Table 1.

As a standard method for the preparation of UFA use was made of the same extraction procedure as described above after evaporation of the purified plasma extract in glass-stoppered test tubes and with a M/15 glycine buffer of pH 12. Also the petroleum ether from extraction with the alkaline ethanol before acidification and containing fatty acid esters was studied for radioactivity.

The possibility of some of the injected labelled fatty acids being recoverable from the red blood cells was studied both after the injection experiment and by the addition of albumin solution with labelled fatty acids to whole blood *in vitro*. Neither in experiments with palmitic acid nor with oleic acid could any activity be demonstrated in the blood cells washed with saline. Calculation of the hematocrit from

Table 1.

Influence of pH on the extraction of fatty acids with petroleum ether from alkaline ethanol.

pH	μ Eq of free fatty acids recovered ¹	Per cent recovered palmitic acid 1-C ¹⁴		Per cent recovered oleic acid 1-C ¹⁴	
		before acidification	after acidification	before acidification	after acidification
10	0.84	5	92	6	90
11	0.83	2	97	1.2	95
12	1.62	1.5	98	0.9	98
13	2.74	1.1	97	1.4	96

¹ Estimated according to GROSSMAN et al. (1955): 0.76 μ Eq.

the determinations of the labelled fatty acids in whole blood and in plasma also agreed well with direct determination of the hematocrit.

All quantitative determinations of UFA were carried out by the technique of GROSSMAN et al. (1955).

A systematic error is unavoidable owing to incomplete mixing of the injected solution with the circulating plasma volume because of the short duration of the experiment. In order to determine the significance of this error one experiment (experiment 1) was carried out in which J¹³¹-labelled human albumin (4 μ C) was added to the injection solution with labelled palmitic acid the J¹³¹ activity was determined on a series of samples.

Isotope technique. The isotope measurements were carried out with a windowless gasflow GM-tube (Tracerlab). The background was about 30 c. p. m. At least 1,000 counts were counted throughout. All samples with free fatty acids were plated directly on aluminium planchets after having been dissolved in ethanol and neutralised with 0.02 N NaOH. The load per planchet (surface 4.7 cm²) never exceeded 0.2 mg/cm². Probably because the plating was seldom ideal, a self absorption loss of about 5 per cent was, however, noted (calculated from the results of experiments with addition of labelled fatty acids to fatty acids prepared from serum). In the determination of radioactivity of esterified fractions of the fatty acids loads of 1 and 1.5 mg per cm² occurred and the self-absorption then noted was about 20–30 per cent. This appeared negligible, because the activity of these fractions never exceeded about 1 per cent of the amount administered and thus did not influence the calculation of the amount of fatty acids eliminated from the blood stream.

In the experiments with J¹³¹-labelled albumin isotope determinations were made on 3 ml samples in a Well type scintillation counter (Tracerlab). Background about 300 c. p. m., 20,000 impulses were counted throughout.

Results and discussion.

Injection of the unesterified fatty acids in albumin-bound form was selected in order to mimic conditions prevalent in plasma. Although the association constants for interaction between higher, undialysable fatty acids with albumin have not been determined, it is probable that albumin can bind at least 7 mol oleic acid per mol and possibly somewhat less palmitic acid (DAVIS and DUBOIS 1946, COGIN and DAVIS 1951, GORDON 1955). The albumin used originally contained 1.8 mol fatty acid per mol albumin and the addition of labelled palmitic and oleic acid in the amounts used resulted in a final ratio of 3.8 and 3.5 mol per mol albumin, respectively. The albumin can thus be considered as roughly half saturated with fatty acids. Therefore there is no reason to expect a deviating disappearance rate due to any excess of fatty acids not bound to albumin.

That the unesterified fatty acids administered have not any appreciable influence on the total amount of UFA in plasma is obvious, because only about 0.032 mEq was injected, while the total amount of UFA in plasma in the experiments varied between 6.6 and 0.81 mEq.

The results of the experiments are apparent from Figs. 1—4. The concentration of UFA in plasma was assumed to be constant during the periods covered by collection of the blood samples. In order to facilitate comparison between the different experiments, all radioactivity values were converted to per cent of the predicted value expected after dilution of the injected fatty acids in the plasma volume. This in turn was assessed as 4.5 per cent of body weight (v. PORAT 1951).

In view of the short interval between the injection of the labelled fatty acids and the sampling, the time for complete mixing of the fatty acids administered with the circulating plasma volume must be taken into account. BERSON and YALOW (1955) found in patients without impairment of circulation that injected J^{131} -labelled albumin appears in a concentration 10 per cent higher in the arterial blood after 1—1.5 minutes than after mixing with the entire circulating blood volume, which requires 5—10 minutes. This also appears to be the case in the present investigation, in which after about 2.7 minutes 3 per cent, after 4.6 minutes 2 per cent, and after 8.3 minutes 1 per cent higher concentration of J^{131} -labelled albumin was noted than after 20 minutes.

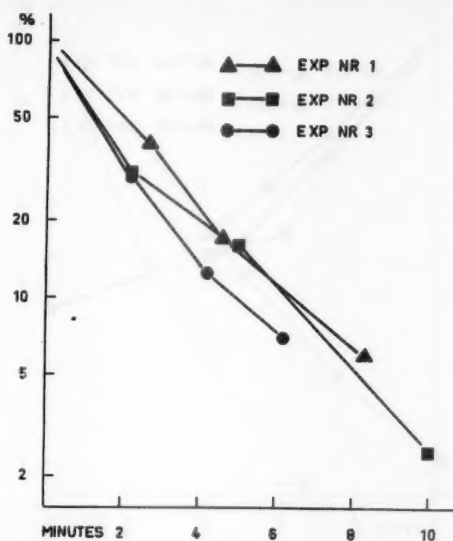


Fig. 1. Disappearance rate of palmitic acid. Post absorptive.

Exp. 1: UFA 0.47 mEq/l, hours since last meal 15.

" 2: " 0.74 " " " " " 12.5.

" 3: " 0.99 " " " " " 14.

It therefore appears that no appreciable error is to be expected from incomplete mixing of the labelled fatty acids and the plasma volume at the time the samples were collected.

However, some curves especially in the oleic acid experiments, showed a more rapid disappearance of the labelled fatty acids immediately after the injection than later. This might be due to a more rapid uptake of the UFA during the first passage of the labelled fatty acids through the circulation, when the administration of the labelled fatty acids can result in a temporary increase in the total concentration of UFA. That UFA in plasma consist mainly of palmitic and oleic acid is apparent from DOLE's (1956 b) determinations. Although his results permit no conclusion as to the exact ratio between oleic and palmitic acid, oleic acid appears to be the larger fraction.

From Figs. 1—4 the half life in plasma of the fatty acids used was determined approximatively. For palmitic acid a half life of about 1.7 minutes was noted after the administration of

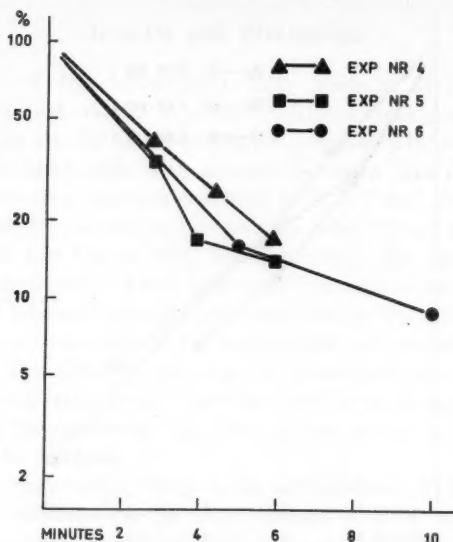


Fig. 2. Disappearance rate of palmitic acid. Starving.

Exp. 4: UFA 0.98 mEq/l, hours since last meal 38.

" 5: " 1.51 " " " " " 60.

" 6: " 1.68 " " " " " 68.

glucose and of about 2 minutes during the post absorptive state. In the experiments with prolonged fasting the curves were not so linear, and the half life could not be determined with accuracy but might be between 2.5 to 5 minutes.

The experiments with oleic acid gave more satisfactory curves

Table 2.

Calculations on data obtained in the experiments with oleic acid 1-C¹⁴.

Exp. no.	Conditions	Plasma volume l	UFA mEq/l	Circulating UFA total g ¹	Half life min.	g UFA turned over/24 hours	Equivalent calories
9	Fasting	4.2	0.42	0.50	3.1	116	1,080
10	Starving	3.1	2.15	1.90	3.8	360	3,350
11	Post glucose..	2.9	0.62	0.50	2.7	133	1,240

¹ Molecular weight calculated as 280.

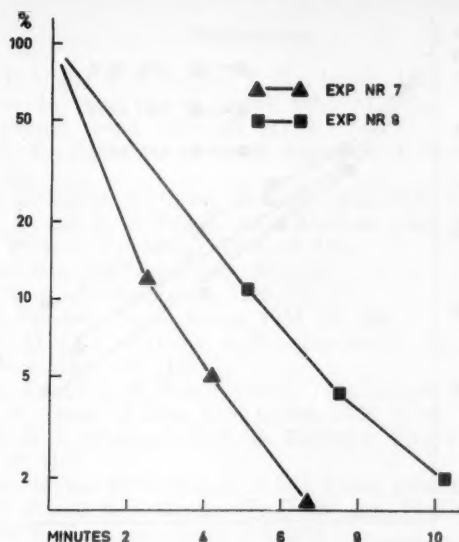


Fig. 3. Disappearance rate of palmitic acid. After ingestion of glucose.

Exp. 7: UFA 0.31 mEq/l.

Exp. 8: UFA 0.27 mEq/l.

with the same tendency to a lower disappearance rate during fasting and a more rapid disappearance rate after the administration of glucose. The data obtained in these experiments are summarised in Table 2.

Such calculation is, of course, only approximative because little is known of the effect of physical activity or of any diurnal variation in the turnover of UFA in plasma. Neither were the experiments carried out under strictly basal conditions. Nevertheless, the results suggest that the increased amount of lipids transported in the form of UFA in the circulation during starvation is sufficient to cover the bulk of the caloric requirements during this state. This, however, requires the assumption that the transport occurs mainly in one direction from fat depots to tissues needing a supply of energy.

It is as yet not known whether fatty acids may also be transported from the fat depots as glycerides, and it is possible that during starvation UFA may to some extent represent fatty acids retransported from plasma glycerides as is the case during removal

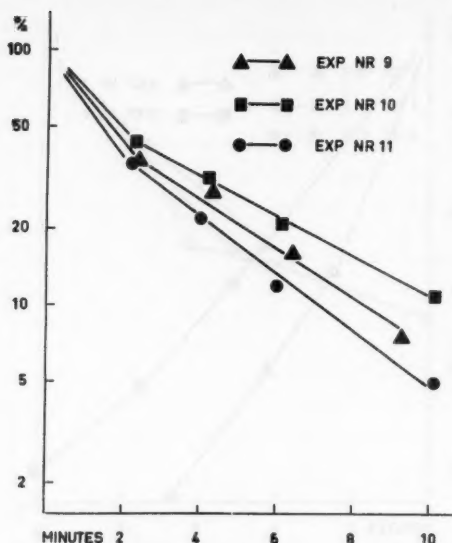


Fig. 4. Disappearance rate of oleic acid.

Exp. 9: UFA 0.42 mEq/l, hours since last meal 13.
 " 10: " 2.15 " " " " " 63.
 " 11: " 0.62 " after ingestion of glucose.

of chylomicrons from plasma (HAVEL and FREDRICKSON 1956). However, WADSTRÖM's (1957) demonstration of increasing amounts of lower glycerides in subcutaneous fat after administration of epinephrine argues strongly for the assumption that fatty acids are transported directly from fat depots as UFA.

Summary.

The disappearance rate of unesterified palmitic acid and oleic acids in plasma in man was studied after varying periods of fasting and after the administration of glucose. Although the biologic half life increased during prolonged fasting, the turnover was large enough to satisfy the bulk of the caloric requirements, at least after 3 days' fasting.

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Effects of Histaminase Inhibitors on the Blood Pressure Responses in Dogs to Histamine Injected into the Renal Artery.

By

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The capacity of the perfused canine kidney to inactivate histamine was demonstrated by BEST and MCHENRY in 1930. They gave the name histaminase to the enzyme responsible for the inactivation of histamine. ZELLER (1951) found that histaminase acted on several diamines and proposed that the enzyme should be called diamine oxidase. Recently DAVISON (1956) presented further evidence consistent with the view that histaminase and diamine oxidase from pig kidney are identical. In the present study the name histaminase is used as has been the custom in earlier publications from this laboratory. SCHAYER (1952 and 1953) using C^{14} histamine demonstrated the action on histamine of another enzyme system, which he called "histamine metabolizing enzyme II". ZELLER, STERN and BLANKSMA (1956) found evidence for the oxidation in vitro of histamine by an enzyme which is not diamine oxidase.

The role, if any, played by these various enzymes in the inactivation of histamine in the kidney of the living dog is not known.

The present experiments were made with the intention of gaining information about the role of the renal histaminase in the removal of histamine injected into the blood stream of dogs. A technique in principle similar to that of EMMELIN (1951) and LINDELL and WESTLING (1956) was used. Histamine was injected into the renal artery. The histamine which escaped into the general circulation caused a fall in blood pressure. This fall was compared

with the fall produced by histamine injected by vein. Histaminase inhibitors potentiated the depressor responses to histamine injected into the renal artery but not those to histamine injected into the femoral artery or given by vein. To obtain more information about the mechanism of action of the inhibitors their effects on the depressor responses to some histamine analogues were studied. No potentiation was observed. In some preliminary studies with the Warburg manometric technique it was found that these histamine analogues with histamine-like action were not oxidized by crude histaminase preparations from canine kidneys.

Methods.

The general plan for the experiments was as follows:

I. *Experiments with the Warburg Manometric Technique.*

- A. Studies on the oxidation of some histamine analogues by crude enzyme preparations from canine kidneys.
- B. Studies on the capacity of some histaminase inhibitors, added in vitro, to inhibit the oxidation of histamine by crude enzyme preparations from canine kidneys.

II. *Studies in Anaesthetized Dogs.*

- A. The blood pressure responses to histamine injected by vein or into the femoral or renal artery.
- B. The blood pressure responses to histamine analogues injected by the same routes.
- C. The effects of histaminase inhibitors on the depressor responses to histamine injected 1) by vein, 2) into the femoral artery, 3) into the renal artery.
- D. The effects of histaminase inhibitors on the depressor responses to histamine analogues injected into the renal artery.
- E. The effects of the injected histaminase inhibitors on the activity of crude histaminase and amine oxidase preparations from the kidneys removed at the end of the experiments.

Drugs used: Histamine acid phosphate, 2- β -aminoethyl pyridine (here called pyridylethylamine), 3- β -aminoethyl-1,2,4-triazole (here called triazole). The doses for these substances when injected are given in μ g base per kg b. wt. per min. Aminoguanidine sulphate, 2-methyl-4-amino-5-aminomethyl-pyrimidine dihydrochloride (B_1 -pyrimidine) and isonicotinyldiazine base were used as inhibitors. Injected doses in mg per kg b.wt. In the Warburg experiments the following drugs were also used: cadaverine dihydrochloride, L-p-sympatol hydrochloride and tyramine acid phosphate.

Enzyme studies: The Warburg technique for measuring oxygen uptake was used as described by LINDELL and WESTLING (1957). For the experiments in group I: A and B kidneys from dogs not given histaminase inhibitors were used. For the experiments in group II: E the kidneys were taken from dogs used in the other experiments of group II.

The enzyme preparations were made in the following way. The kidneys which had been stored at -15°C were cut with scissors. 10 g of the tissue were added to 20 ml 0.067 M phosphate buffer (pH 7.4) in a homogenizer and ground for 30 minutes. Cooling of the homogenizer by running tap water kept the temperature of the homogenate below 15°C . 12 ml of the homogenate were pipetted off to be used as a crude amine oxidase preparation, *i. e.* it oxidized tyramine and sympatol. The remainder was centrifuged (3,000 r.p.m.) at room temperature for 10 minutes. The supernatant was used as a crude histaminase preparation (the uncentrifuged homogenate oxidized histamine and cadaverine too, but did so at a slower rate than the supernatant). The substrates, histamine, pyridylethylamine, sympatol, tyramine and cadaverine, were dissolved in distilled water and when necessary, neutralized with NaOH. The initial concentration after tipping of histamine and cadaverine, was calculated to be 0.002 M and of the other substrates 0.01 M. With these concentrations of sympatol, pyridylethylamine and cadaverine the oxygen uptake proceeded at a steady rate for the first 20 minutes and the readings taken during this time were used for the calculation of the oxygen uptake. With histamine the oxygen uptake was approximately linear for the first 60 minutes and the readings obtained during this time were used for the calculations. In the experiments of group I the inhibitors were added to the enzyme preparation in the main compartment of the Warburg vessel. The substrates (or distilled water for enzyme blanks) were added 30 minutes later. The enzyme blanks contained the same amount of inhibitor as did the vessels to which substrates were added.

Experiments in anaesthetized dogs: 11 female and 9 male dogs were used.

Anaesthesia: Sodium pentobarbitone (Nembutal Abbot) was given by vein. The initial dose was usually 30 mg per kg b.wt. It is known that anaesthesia may influence the histamine sensitivity (FELDBERG and SCHILF 1930, WYMAN, DRAPEAU and FULTON 1956). In the present experiments the anaesthesia was adjusted so that the eye lid reflex was abolished, but the capacity to breathe spontaneously was maintained. This could usually be obtained by a continuous infusion of sodium pentobarbitone, 15–20 per cent of the initial dose being given per hour.

Operative procedures: The dogs were intubated endotracheally. A polythene catheter was inserted into one of the renal arteries from a femoral artery as described by LINDELL and OLIN (1957). In experiments where the abdomen was opened the catheterization was made easier by placing a metal indicator at the level of the renal artery. The position of the catheter was verified at the end of the experiments. A polythene tube of the same dimensions as that used for the renal artery was inserted into the inferior caval vein from one of the femoral veins. These two polythene tubes were used for the injection of histamine or histamine analogues. Two more polythene tubes were tied into suitable veins in the legs for the administration of anaesthesia and inhibitors. In some animals at the beginning of the experiment one

kidney was removed through a midline abdominal incision. At the end of every experiment the catheterized kidney was removed. The kidneys were weighed and stored at -15°C until used for the enzyme studies described above. The blood pressure in a carotid artery was recorded with an ordinary mercury manometer. The rectal temperature was maintained at approximately 38°C by application of radiant heat from ordinary light bulbs.

Injections: Histamine and histamine analogues were injected with motordriven syringes, which were automatically or semiautomatically operated. Injection apparatuses of the type manufactured by the institute's workshop were used. This apparatus has an electric motor which can be made to drive a syringe at different rates. The rates can be selected with a 10 speed gear box. The syringes, 20 ml Interchangeable Luer Lock (Summit) were connected to the polythene tubes with suitable needles of stainless steel. Leakage in the syringes was prevented by Silicone grease. The injection apparatuses were started and stopped by an electric timing device. A suitable injection time was found to be 24 seconds. The intervals between the injections were usually 108 to 216 seconds. The volumes delivered at different gear ratios by the motordriven syringes, connected to the polythene tubes used in the actual experiments were collected and measured. There was a certain variation in the volume delivered with one and the same gear ratio, especially when the ratio had just been changed. With the volumes used here (usually 0.4–2.5 ml) the greatest variation was seen with a ratio that gave 0.46 ± 0.006 ml in 24 seconds; the extreme values being 0.45 and 0.50 ml. Making the injection against pressures up to 200 mm of mercury did not affect the delivered volumes.

In two preliminary experiments with anaesthetized dogs it was found that under the conditions described above the relations between responses to histamine given into the renal artery and into the caval vein remained unchanged for at least three hours, if no inhibitor was given.

I. Experiments with the Warburg Manometric Technique.

A. Studies on the oxidation of some histamine analogues by crude enzyme preparations from canine kidneys.

Histamine analogues with histamine-like actions were first used by ARUNLAKSHANA, MONGAR and SCHILD (1954) in the elucidation of the potentiating action of histaminase inhibitors. In a study on the oxidation of some histamine analogues in vitro (LINDELL and WESTLING 1957) it was found that pyridylethylamine and triazole were not oxidized by histaminase preparations from pig and cat kidney. Evidence was found that pyridylethylamine was oxidized by amine oxidase preparations from guinea pig and rabbit liver and cat kidney. In view of known species dif-

ferences between amine oxidases (BLASCHKO, CHATTERJEE and HIMMS 1955 and BLASCHKO and HIMMS 1955) it was desirable to study the behaviour of histaminase and amine oxidase preparations from canine kidneys towards pyridylethylamine and triazole before using these substances in investigating the action of histaminase inhibitors in dogs.

Triazole was not oxidized by crude enzyme preparations from canine kidneys which oxidized cadaverine and histamine as well as tyramine and sympatol. Triazole in a concentration of 0.01 M did not affect the oxygen uptake by these enzyme preparations in the presence of the above mentioned amines.

The addition of pyridylethylamine to a crude kidney homogenate caused an oxygen uptake, which was not affected by aminoguanidine in a concentration of 10^{-4} M which completely inhibited the oxidation of cadaverine and histamine. It thus seems unlikely that pyridylethylamine was oxidized by the enzyme which oxidized cadaverine and histamine. When pyridylethylamine was added together with sympatol or tyramine to an amine oxidase preparation from canine kidney the oxygen uptake was not additive but intermediate between that of pyridylethylamine itself and that caused by tyramine or sympatol. This may be taken to indicate that pyridylethylamine was oxidized by amine oxidase. These results are in agreement with those of LINDELL and WESTLING (1957).

B. Studies on the capacity of some histaminase inhibitors added in vitro to inhibit the oxidation of histamine by crude enzyme preparations from canine kidneys.

The histaminase inhibitors used in the present investigation were aminoguanidine, B_1 -pyrimidine and isonicotinylhydrazine. Such inhibitors have been used by among others SCHAYER, KENNEDY and SMILEY, 1953; ARUNLAKSHANA, MONGAR and SCHILD 1954; WESTLING 1956. Before using these inhibitors in the present experiments on anaesthetized dogs, their inhibition of the histaminase from canine kidneys was studied with the Warburg manometric technique. The results are shown in table 1. Aminoguanidine was the strongest and isonicotinylhydrazine the weakest of the three inhibitors studied in this way. With suitable concentrations of these inhibitors it was possible to suppress completely the oxygen uptake caused by addition of histamine to crude enzyme preparations from canine kidneys.

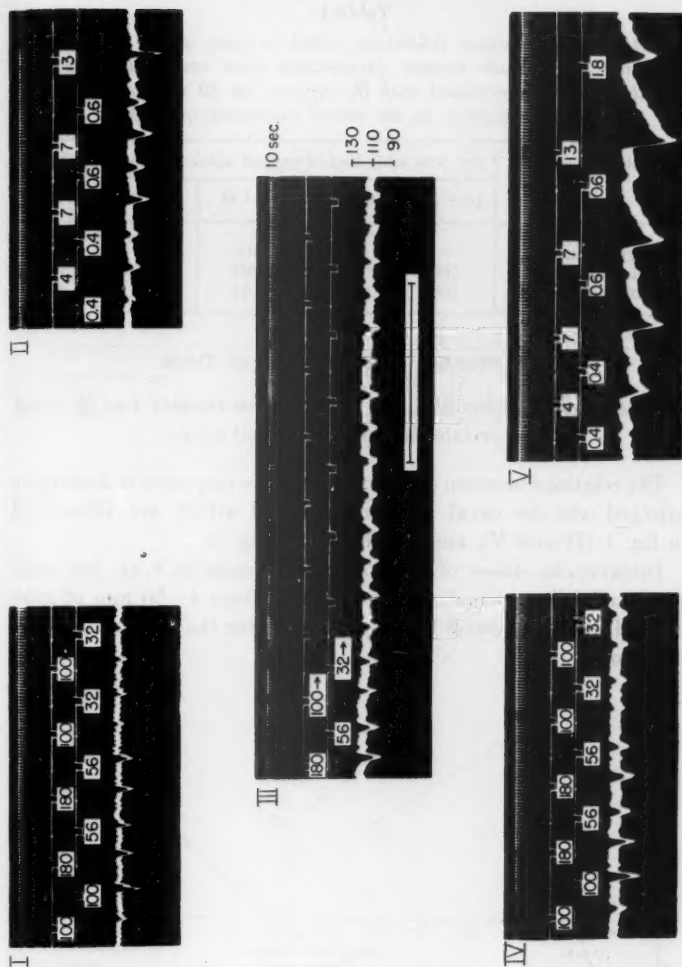


Fig. 1. Dog 10. From above: time in 10 seconds, injections into the renal artery, injections into the caval vein, blood pressure in a carotid artery. Sections I, III, and IV: injections of pyridylethylamine, Section II and V injections of histamine. —•— indicates infusion by vein of aminoguanidine 12.5 mg/kg b.wt. Between sections I and II as well as between IV and V there were pauses of 15 minutes.

Table 1.

The effect of histaminase inhibitors, added *in vitro*, on the oxidation of histamine by a crude enzyme preparation from canine kidneys. The inhibitors were preincubated with the enzyme for 30 minutes, before the addition of histamine in an initial concentration of 0.002 M.

Inhibitor	Per cent inhibition of oxygen uptake by the inhibitor				
	10 ⁻³ M	10 ⁻³ M	10 ⁻⁴ M	10 ⁻⁵ M	10 ⁻⁶ M
Aminoguanidine	—	100	100	82	60
B ₇ -pyrimidine	100	100	81	40	19
Isonicotinylhydrazine . .	100	78	64	30	—

II. Studies in Anaesthetized Dogs.

A. The blood pressure responses to histamine injected into the caval vein or into the femoral or renal artery.

The relations between doses and depressor responses to histamine injected into the caval vein or the renal artery are illustrated in fig. 1 (II and V), and graphically in fig. 2.

Intravenous doses of 0.4–7 μ g histamine/kg b.wt. per min. produced falls in blood pressure ranging from 4–50 mm of mercury. The fall occurred 12–20 seconds after the beginning of the injection.

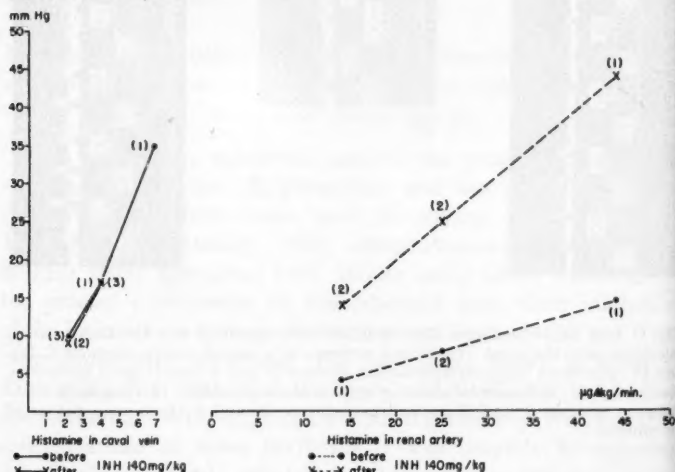


Fig. 2 a.

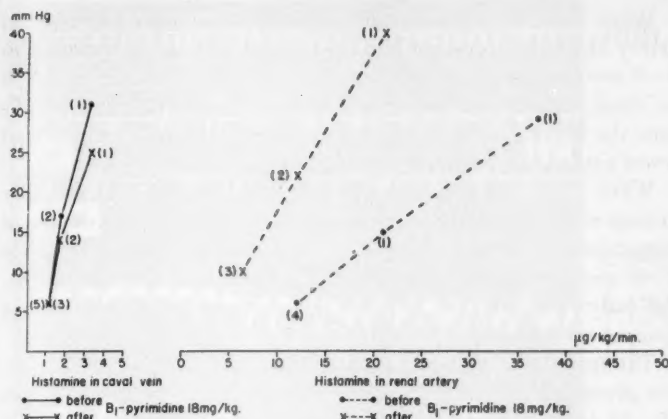


Fig 2 b.

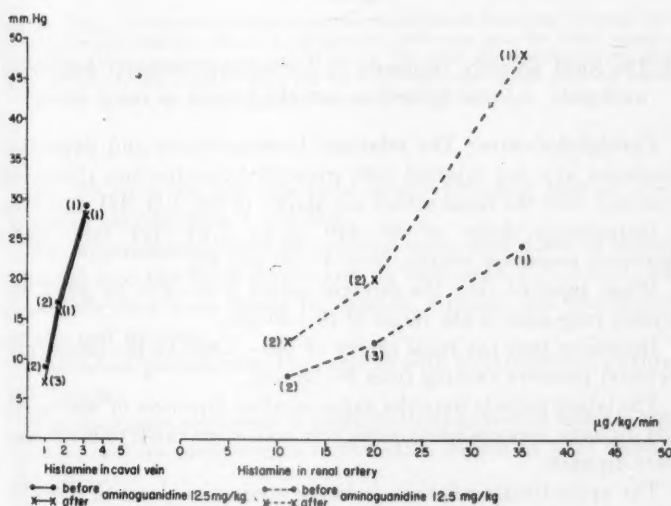


Fig. 2 c.

Fig. 2. Illustrates graphically the relations between doses of histamine and depressor responses in three different dogs. Ordinata: fall in blood pressure in mm of mercury. Abscissa: dose of histamine in $\mu\text{g/kg/min}$.

a) before and after the infusion by vein of isonicotinyldiazine 140 mg/kg b.wt. (the figures within brackets denote the number of observations).

b) before and after the infusion by vein of B_1 -pyrimidine 18 mg/kg b.wt.

c) before and after the infusion by vein of aminoguanidine 12.5 mg/kg b.wt.

When 1.0—4.7 μg histamine were injected into the femoral artery the blood pressure fell 5—34 mm. The latent period was 5—6 seconds longer than after injections by vein. An early fall in blood pressure was sometimes seen after injection of histamine into the femoral artery. The fall occurred within 10 seconds and never exceeded 4 mm.

When 4—80 μg histamine were injected into the renal artery of dogs which had not received histaminase inhibitors the depressor responses were in the range of 4—42 mm. The latent period was 2—4 seconds longer than after injections into the caval vein. An early response such as after injections of histamine into the femoral artery was not observed.

The approximate relations between equiactive doses of histamine given into the renal artery and into the caval vein were 10—15 to 1. The same relations for histamine injected into the femoral artery and by vein were 1—1.5 to 1.

B. The blood pressure responses to histaminase resistant histamine analogues, injected by vein or into the femoral or renal artery.

Pyridylethylamine: The relations between doses and depressor responses in a dog injected with pyridylethylamine into the caval vein and into the renal artery are shown in fig. 1 (I, III and IV).

Intravenous doses of 30—470 $\mu\text{g}/\text{kg}$ b.wt. per min. gave depressor responses ranging from 4—30 mm Hg.

When injected into the femoral artery 160—470 μg gave depressor responses in the range of 10—30 mm.

Injections into the renal artery of 100—1,600 μg produced falls in blood pressure varying from 6—38 mm.

The latent periods were the same as after injection of histamine and diphasic depressor responses were seen when those to histamine were diphasic.

The approximate relations between equiactive doses of pyridylethylamine injected into the renal artery and into the caval vein were 3—4 to 1. The same relations for pyridylethylamine injected into the femoral artery and by vein were 1—1.5 to 1.

Triazole: Intravenous doses of 25—99 μg per kg b.wt. per min. gave depressor responses ranging from 4—20 mm Hg.

25—90 μg injected into the femoral artery produced falls in blood pressure in the range of 4—16 mm.

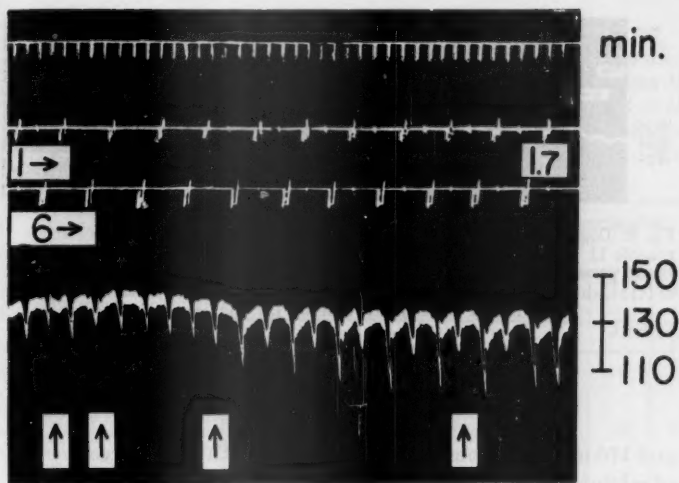


Fig. 3. Dog 1. From above: time in minutes, injections of histamine into the caval vein (numbers indicate doses in $\mu\text{g/kg/min}$), injections into the renal artery of histamine, blood pressure in a carotid artery. The first arrow indicates the injection of 0.0013, the second 0.013, the third 0.13 and the fourth 0.65 mg aminoguanidine per kg b.wt.

11–99 μg given into the renal artery produced falls in blood pressure varying from 4–20 mm.

The approximate relations between equiactive doses of triazole injected into the renal artery and by vein were 1–1.5 to 1. The same relations were found for triazole injected into the femoral artery and by vein.

The latent periods were the same as with histamine and pyridylethylamine.

The depressor responses to pyridylethylamine and triazole were antagonized by mepyramine in the same degree as were those to histamine.

C. The effects of histaminase inhibitors on the blood pressure responses to histamine injected:

1) By vein: In no instance was a potentiation observed. This is illustrated graphically in fig. 2. The greatest amount of inhibitor used was 125 mg/kg of aminoguanidine, 21 mg/kg of B_1 -pyrimidine

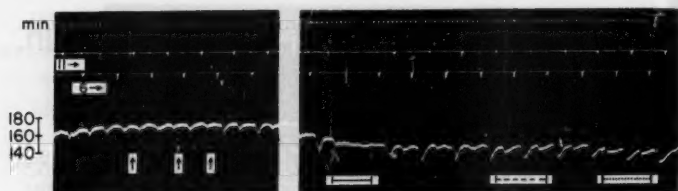


Fig. 4. Dog 4. From above: time in minutes, injections into the renal artery of triazole 11 $\mu\text{g/kg/min}$, injections into the renal artery of histamine 6 $\mu\text{g/kg/min}$. Blood pressure in a carotid artery. First arrow indicates the injection by vein of 0.137, the second 1.37 and the third 13.7 mg isonicotinyldiazine per kg b.wt. | indicates infusion by vein of 137 mg isonicotinyldiazine per kg b.wt. | indicates infusion of aminoguanidine 10 mg/kg b.wt. | indicates infusion of B_1 -pyrimidine 10 mg/kg b.wt.

and 176 mg/kg of isonicotinyldiazine. With these large amounts of inhibitors there was a tendency towards a decrease in sensitivity to histamine given by vein.

In some experiments (dogs 6, 8, 9, 10, 12, 16) the effects of the inhibitors on the depressor responses to pyridylethylamine or triazole given by vein were studied. In no instance was a potentiation observed. The relations between equiactive doses of histamine and histamine analogues given by vein were not changed by the inhibitors.

2) Into the femoral artery. One experiment with each of the inhibitors was made. The doses were: aminoguanidine 125 mg, B_1 -pyrimidine 21 mg and isonicotinyldiazine 176 mg/kg. No potentiation was observed.

3) Into the renal artery. The depressor responses to histamine injected into the renal artery were regularly potentiated after the administration of inhibitors in suitable amounts.

In the present investigation the inhibitors were always given by vein, and usually in the form of prolonged infusions. When the experiment was aimed at finding the smallest potentiating dose the inhibitor was given in single intravenous injections.

Aminoguanidine: Under the present experimental conditions the smallest potentiating dose of aminoguanidine was 0.013 mg/kg or 0.1 micromole/kg (fig. 3). The potentiation was nearly maximal when 0.13 mg/kg had been given; that is no further increase in potentiation was seen after a fivefold increase in the dose of the inhibitor. The potentiation caused by 12.5 mg aminoguanidine/kg

Table 2.

Oxygen uptake by crude renal amine oxidase and histaminase preparations from dogs submitted to the usual experimental procedures, including the administration of inhibitors. The kidneys were removed at the end of the experiments. The substrates were sympatol = *S* 0.01 M, cadaverine = *C* 0.002 M and histamine = *H* 0.002 M. *A* = aminoguanidine, *B* = *B*₁-pyrimidine and *I* = isonicotinyldiazine.

Dog No.	Sex	B.wt. kg	Inhibitor mg/kg b.wt.	Time elapsed between the administration of the inhibitor and the removal of the kidney min.	Oxygen uptake μ l/g/hr		
					<i>S</i>	<i>C</i>	<i>H</i>
1	♀	10	<i>A</i> 12.5	8	67	0	0
4	♀	10	<i>I</i> 137.0	10	77	0	0
			<i>A</i> 10.0				
			<i>B</i> 10.0				
5	♀	10	<i>B</i> 21.0	10	83	0	0
			<i>A</i> 12.5				
			<i>I</i> 40.0				
6	♀	11	<i>A</i> 12.5	140	120	0	0
11	♂	26	<i>A</i> 12.5	5	57	3	0
12	♂	8	<i>A</i> 125.0	150	67	0	0
13	♂	14	<i>I</i> 140.0	30	—	0	0
14	♂	17	<i>B</i> 18.0	60	60	30	17
15	♂	18	<i>A</i> 12.5	15	50	0	0
			<i>B</i> 11.0				
16	♀	10	<i>A</i> 12.5	120	57	0	0
17	♀	7.5	<i>A</i> 12.5	not noted	—	—	0
19	♂	12	<i>A</i> 12.5	180	—	0	0

could not be increased further by the administration of 11 mg *B*₁-pyrimidine/kg (dog 15). The potentiating effect of 12.5 mg aminoguanidine/kg lasted at least 2½ hours which is the longest observation period in this investigation.

*B*₁-pyrimidine: The smallest potentiating dose of *B*₁-pyrimidine in these experiments was 0.21 mg/kg or 1 micromole/kg and the potentiation was nearly maximal when 2.1 mg/kg had been given. The potentiation caused by 21 mg *B*₁-pyrimidine/kg was not further increased by the administration of aminoguanidine 12.5 mg/kg, or isonicotinyldiazine 40 mg/kg (dog 5).

Isonicotinyldiazine: The smallest potentiating dose in these experiments was 13.7 mg/kg or 100 micromoles/kg (fig. 4). The potentiation was maximal when 137 mg/kg had been given. The subsequent administration of aminoguanidine 10 mg/kg or *B*₁-pyrimidine 10 mg/kg did not increase the degree of potentiation.

It may be seen from the figures for the smallest potentiating dose of the inhibitors and from table 1 that there was a relationship

Table 3.

Oxygen uptake by crude renal amine oxidase and histaminase preparations from three dogs submitted to the usual experimental procedures. Observations are given for kidneys removed at the beginning of the experiments and for kidneys removed after the administration of inhibitors. The substrates were sympathol = S 0.01 M, pyridylethylamine = P 0.01 M, cadaverine = C 0.002 M and histamine = H 0.002 M. A = amino guanidine, B = B₁-pyrimidine, I = isonicotinyldiazine.

Dog No.	Sex	B.wt. kg	Kidney removed at the beginning of the experiment. Oxygen uptake $\mu\text{l/g/hr}$			Wt. of kidney g	Inhibitor mg/kg b.wt.	Time elapsed between administration of inhibitor and removal of kidney min.	Kidney removed at the end of the experiment. Oxygen uptake $\mu\text{l/g/hr}$			Wt. of kidney g
			S	P	H				S	P	H	
8	♂	8	47	—	190	17	I 176.0	60	50	—	0	19
9	♀	7	117	40	143	14	B 21.0	65	103	50	0	15
10	♂	11	120	—	110	50	A 12.5	150	73	—	7	45

Table 4.

Oxygen uptake by crude renal amine oxidase and histaminase preparations from dogs which were not given inhibitors but were otherwise submitted to the usual experimental procedures. The substrates were sympatol = S 0.01 M, pyridylethylamine = P 0.01 M, cadaverine = C 0.002 M and histamine = H 0.002 M.

Dog No.	Sex	B.wt. kg	Kidney removed at the beginning of the experiment. Oxygen uptake $\mu\text{l/g/hr}$				Wt. of kidney g	Kidney removed at the end of the experiment. Oxygen uptake $\mu\text{l/g/hr}$				Wt. of kidney g
			S	P	C	H		S	P	C	H	
2	♂	16	137	—	157	82	31	113	—	90	67	41
3	♀	10	120	—	144	67	15	90	—	73	31	20
7	♀	21	80	43	170	82	30	67	43	110	40	45
17	♀	9	not removed				—	70	40	87	31	18

between the potentiating capacity *in vivo* and the inhibiting capacity *in vitro*.

The doses required for potentiation here are of the same order of magnitude as those required to potentiate the action of histamine in unanaesthetized guinea pigs (WESTLING, 1956).

The degree of potentiation at different dose levels of histamine is shown graphically in fig. 2. The increase in the dose of histamine was produced by increasing the volume of histamine solution injected. The actual blood pressure record from an experiment is shown in fig. 1.

The greatest degree of potentiation obtained in these experiments was such that the average relations between equiactive doses of histamine given into the renal artery and into the caval vein were 4—6 to 1.

D. The effects of histaminase inhibitors on the blood pressure responses to histaminase resistant histamine analogues injected into the renal artery.

The effects of the inhibitors on the depressor responses to pyridylethylamine or triazole injected into the renal artery were studied in seven dogs (4, 5, 6, 10, 15, 16, 19). In no instance was a potentiation observed.

E. The effects of the injected inhibitors on the activity of crude histaminase and amine oxidase preparations from kidneys removed at the end of the experiments.

When an experiment was finished the catheterized kidney was removed and treated as described under methods. The histaminase preparations made from these kidneys were incubated with histamine and cadaverine. For comparison amine oxidase preparations from the same kidneys were incubated with sympatol, a typical amine oxidase substrate, and sometimes also with pyridylethylamine. The oxygen uptake caused by the addition of these amines to the enzyme preparations is shown in table 2.

In some experiments one kidney was removed at the beginning of the experiment and the remaining kidney removed at the end of the experiment. A comparison of the enzyme activities of these kidneys is shown in table 3. It may be seen from these tables that under the prevailing experimental conditions the inhibitors completely or nearly completely suppressed the oxygen uptake normally caused by the addition of histamine or cadaverine to enzyme preparations from canine kidneys. The oxygen uptake caused by the addition of sympatol or pyridylethylamine did not seem to be affected by the injected inhibitors. In dogs subjected to the same experimental procedure as those described above with the exception that no inhibitor was given, the enzyme preparations from the kidney removed at the end of the experiment took up less oxygen when incubated with histamine and cadaverine than did those from the kidney removed at the beginning of the experiment, table 4. The reason for this is not known.

Discussion.

The results of the experiments with the Warburg manometric technique indicate that the histaminase and amine oxidase of canine kidney behave in the same way towards pyridylethylamine and triazole in vitro as did similar enzyme preparations from cat kidneys (LINDELL and WESTLING 1957). The experiments where histaminase inhibitors were added in vitro to crude enzyme preparations from canine kidneys do not give any evidence for the oxidation of histamine by an enzyme different from histaminase.

In the experiments with anaesthetized dogs the latent period of the depressor response to histamine injected into the renal artery supports the view that this fall was caused by the same factors as was the fall after intravenous injections of histamine. The late depressor response to histamine injected into the femoral artery was probably caused by the same mechanism. A similar conclusion was drawn by LINDELL and WESTLING (1956), working with cats.

The depressor responses to histamine injected into the femoral artery or by vein were not potentiated by the inhibitors used: *i. e.* aminoguanidine, B_1 -pyrimidine and isonicotinyldrazine. These inhibitors regularly potentiated the depressor responses to histamine injected into the renal artery. Under the same conditions the depressor responses to two histamine analogues were not potentiated. Pyridylethylamine (ARUNLAKSHANA *et al.* 1954) and triazole (WHITE 1957) are presumed to act on the same "receptors" as does histamine itself. It thus seems unlikely that the potentiation observed here was due to an action of the inhibitors on the receptors responsible for the fall in blood pressure.

It can be assumed that the potentiation was due to an action of the inhibitors on the kidneys. It is conceivable that an increase in the rate of blood flow through the kidneys or a change in the distribution of this blood flow might reduce the removal of histamine from the blood passing through the renal blood vessels. Such a mechanism should also affect the removal of pyridylethylamine. The blood pressure responses to this histamine analogue were however not potentiated by the inhibitors.

The classical investigations by BEST and MCHENRY have shown that the canine kidney contains histaminase. The inhibitors used in the present study inhibit the oxidation of histamine and cadaverine *in vitro*. It thus seems possible to infer that the effects of the inhibitors, namely the potentiation observed, was due to the inhibition of the oxidation of histamine in the kidney of the living dog. The evidence for this is:

1. The effects of the histamine analogues studied were not potentiated and these substances were not oxidized by histaminase from canine kidney *in vitro*.
2. There was a relationship between the inhibiting capacity *in vitro* and the potentiating capacity *in vivo* of the inhibitors studied.

3. The oxidation of histamine and cadaverine by enzyme preparations from kidneys removed at the end of the experiments were completely or nearly completely suppressed when the animal had received injections of inhibitors in amounts sufficient to potentiate.

4. The oxidation of pyridylethylamine under the same conditions was not materially affected by the injected inhibitors.

When maximum potentiation had been obtained with one inhibitor, the administration of the other inhibitors did not cause any further potentiation. This may be taken to indicate that the inhibitors act through a common mechanism and that the greatest degree of potentiation obtainable was determined by this mechanism and not by the dose of the inhibitor. When maximum potentiation had been obtained by histaminase inhibitors, the kidney still retained a considerable capacity to remove injected histamine as judged from the relations between equiactive doses of histamine given into the renal artery and into the caval vein. It is possible that some of this remaining removal of histamine was due to the action of SCHAYER's "histamine-metabolizing enzyme II". It is also possible that some of the injected histamine was stored temporarily in the kidney (EMMELIN 1951). EMMELIN found that under certain conditions some of the histamine injected into the renal artery of a cat was excreted in the urine. In man, ADAM, CARD, RIDDELL, ROBERTS and STRONG (1954) found that about 1 per cent of histamine given by vein appeared in the urine as free histamine. SCHAYER (1957) found no C^{14} histamine in the urine after subcutaneous injection of labeled histamine in dogs. A comparison of these results requires consideration of doses and ways of administration.

Summary.

1. The effects on the dog's blood pressure of histamine injected by vein or into the femoral or renal artery have been studied.

2. The effects of two histamine analogues, which were not oxidized by histaminase in vitro were also studied under the same conditions.

3. As judged from the relative effectiveness of histamine injected by these routes the kidney had a considerable capacity to remove injected histamine from the circulating blood.

4. Histaminase-resistant histamine analogues were not removed as effectively as histamine by the kidney.
5. Histaminase inhibitors, such as aminoguanidine, B₁-pyrimidine and isonicotinylhydrazine potentiated the depressor responses to histamine given into the renal artery but not those to histamine given into the femoral artery or by vein.
6. The depressor responses to the histaminase-resistant histamine analogues were not potentiated by the inhibitors.

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Fibrinolytic Activity Derived from Guinea Pig Serum by Peptone.

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UNGAR and MIST (1949) and UNGAR, DAMGAARD and HUMMEL (1953) demonstrated fibrinolytic activity in normal guinea pig serum after treatment with peptone followed by isoelectric precipitation. They also reported the development, under similar conditions, of fibrinolytic activity in sensitized guinea pig serum after addition of the corresponding antigen. The fibrinolytic activity was supposed to be caused by the blood protease plasmin (fibrinolysin). UNGAR (1953) has suggested that activation of the fibrinolytic system could be involved in the mechanisms of anaphylaxis and in "anaphylactoid" reactions, including peptone shock.

We have studied the reactions described by UNGAR et al. in order to find out if these phenomena can be correlated with the concept of fibrinolysis as developed in this laboratory (ASTRUP 1956). The purpose of this paper is to present experiments on the effect of peptone upon guinea pig serum. They were undertaken in an attempt to reproduce and extend Ungar's observations with a different technique as a basis for a further elucidation of the reactions involved in the process.

Materials and Methods.

Guinea pig serum was obtained by spontaneous coagulation of blood collected from the carotid arteries or by cardiac puncture. The samples were stored at -20°C . The peptone was (if not otherwise stated) "Bacteriological Peptone", RMC, kindly supplied by Roskilde Medical

Company, Roskilde, Denmark. Bovine plasminogen, prepared according to ASTRUP and STERNORFF (1953), was placed at our disposal by Dr. TAGE ASTRUP.

Serum and peptone solution, made up in distilled water or physiological saline, were mixed and (after a fixed period) diluted with distilled water to 20 times the total volume (if peptone was dissolved in saline) or to 20 times the serum volume (if peptone was dissolved in water). The pH of the diluted serum-peptone mixture was adjusted to 5.3 with 0.5% acetic acid. The precipitate formed was separated by centrifugation and dissolved in a volume of barbital buffer (0.05-M; pH 7.75; 0.1-M NaCl; total ionic strength = 0.15) equal to the original volume of serum. The same buffer was used for preparing dilutions of the original euglobulin solution. Controls were made by substituting saline or distilled water for the peptone solutions.

The euglobulin solutions were examined for fibrinolytic activity by the fibrin plate method (0.1% bovine fibrinogen), described by ASTRUP and MÜLLERTZ (1952). The thrombin used for clotting was kindly supplied by Løvens kemiske Fabrik, Copenhagen. The fibrinolytic activity was recorded as the diameter products (mm²) of the lysed zones after 18 hours' digestion at 37° C (average of 3 determinations). Since the fibrin substrate contains considerable amounts of plasminogen, the recorded fibrinolytic effect is caused either by activators of plasminogen, by an active protease, or by a combination of both. Protease activity was estimated by the heated fibrin plate method, described by LASSEN (1952). The fibrin plates were heated at 85° C for 35 minutes. By this procedure the plasminogen is destroyed and activators of plasminogen will have no effect.

Only the estimations made on the same day and using the same fibrinogen preparation were compared because the sensitivity of the fibrin substrates varied with each lot of fibrinogen. These variations are presumably due to variations in the content of plasminogen and inhibitory agents, depending on the individual differences in the donor animals. The variations in sensitivity are of less significance in the heated fibrin plates, because inhibitory substances are destroyed during the heating procedure. These circumstances probably account for the observations that solutions with low proteolytic activity on heated fibrin plates in some experiments produced no lysis on the standard fibrin plates (containing more inhibitory substances), whereas more potent solutions generally produced the greatest lysis on the standard fibrin plates.

When solutions of peptone (2, 4, or 8%) were added to equal volumes of serum and the mixtures diluted and precipitated after two minutes at room temperature, the samples of guinea pig serum regularly produced demonstrable fibrinolytic activity on both standard and heated fibrin plates. Most of the peptone-free controls showed no activity, but some showed "spontaneous" activity, which was usually considerably lower than the activity obtained in the corresponding samples with peptone. Different concentrations of peptone, either alone or mixed with the inactive euglobulin samples (no isoelectric precipitation)

produced no lysis. Samples of serum showing more than a trace of "spontaneous" activity after precipitation were not used in the experiments reported here.

In some experiments an incubation at 37° C of the serum-peptone mixture, prior to the isoelectric precipitation, increased the fibrinolytic activity of the euglobulin solution. In other experiments no demonstrable differences were present between samples incubated for 5 minutes at room temperature (21° C) or at 37° C. The reasons for these discrepancies have not been found. In order to obtain constant conditions for the reaction, the sera and peptone solutions in the present investigation were heated in a waterbath to 37° C, before they were mixed and incubated at 37° C. The isoelectric precipitations were carried out at room temperature.

Since the commercial peptone preparations might differ in their effects on the fibrinolytic system in guinea pig serum, the potency of the following products was compared: "Bacteriological Peptone", RMC, "Fibrinpepton", RMC, and "Peptone Orthana Special". Identical concentrations of "Bacteriological Peptone", and "Peptone Orthana Special", produced almost the same fibrinolytic activity, whereas "Fibrinpepton" produced only about one fourth of this activity.

Results.

The influence of the amount of peptone was estimated by adding 1 ml solutions of varying peptone concentrations to 0.5 ml samples of pooled serum. The precipitations were carried out 5 minutes later. With increasing amounts of peptone there was an initial increase in activity, which was followed by a decrease (Fig. 1). With large amounts of peptone the activity went down to zero. The highest activities were obtained with 80 mg and 160 mg per ml of serum. Additional experiments with other sera showed a plateau of optimum effect in the range of 60 to 200 mg peptone per ml of serum.

UNGAR and MIST (1949) have reported that maximum activity was obtained after 2 minutes' incubation of the serum-peptone mixture. This was followed by a rapid decrease during the next 15 minutes. In our experiments no appreciable differences were observed by varying the incubation period from 1 to 80 minutes (at 37° C).

The active samples (see Fig. 1) were able to digest the heated fibrin, hence the presence of a protease was evident. The presence of plasminogen activating agents was investigated by addition of bovine plasminogen to an active sample. The results (Fig. 2) show that the addition of plasminogen increased the activity approximately four times. Plasminogen added to the peptone-free

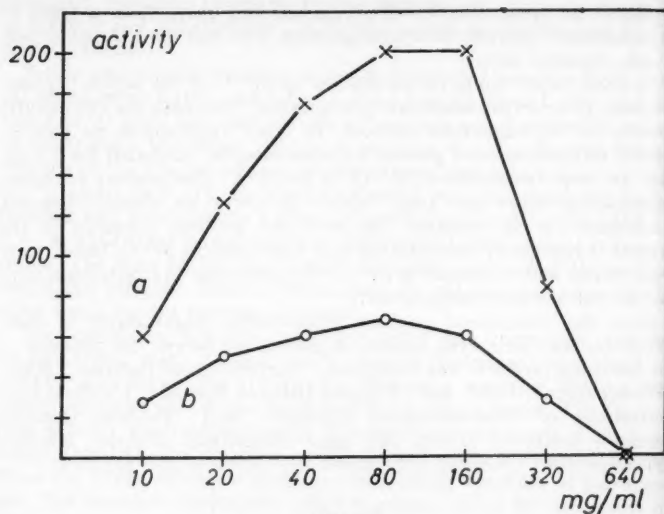


Fig. 1. Different amounts of peptone added to constant amounts of guinea pig serum. Fibrinolytic activity (a) on standard fibrin plates, and (b) on heated fibrin plates. *Abscissa*: mg peptone per ml of serum (logarithmic scale). *Ordinate*: Fibrinolytic activity recorded as diameter product (in mm²) of the lysed zones.

inactive control sample produced no lysis. Increasing amounts of bovine plasminogen produced increasing proteolytic activity on the heated fibrin (Fig. 3). The plasminogen preparation alone, dissolved in barbital buffer to the same concentrations, produced no lysis of either standard or heated fibrin plates. These results demonstrate the presence in the precipitate of an agent capable of transforming bovine plasminogen into plasmin; *i. e.* a plasminogen activator.

Discussion.

Our results have demonstrated that the euglobulin fraction of guinea pig serum (prepared by isoelectric precipitation at 5.3 and low ionic strength) normally shows no fibrinolytic activity on fibrin plates. The addition of peptone to serum before the precipitation, results in the development of fibrinolytic activity, and there is a maximum in the yield of fibrinolytic activity within a certain range of peptone concentration (mg per ml serum).

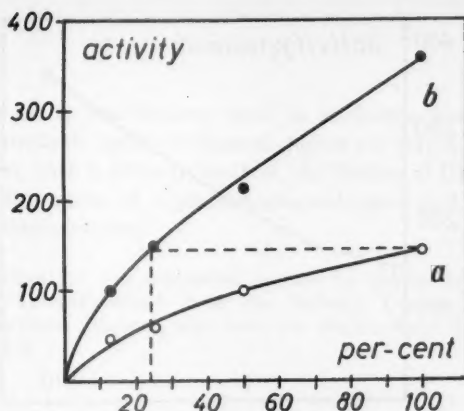


FIG. 2. Effect of bovine plasminogen on protease activity. Activity on heated fibrin plates recorded for (a): precipitate of serum-peptone mixture, and (b): same precipitate with added plasminogen (20 mg per ml). *Abcissa*: Concentration in per cent of original solution. *Ordinate*: Activity.

A proteolytic enzyme and a plasminogen activator are formed by the interaction of serum and peptone. Since the protease is formed from constituents of the blood we call it plasmin, with the reservation that we do not know whether or not it is identical with plasmin activated by other methods. The plasminogen activating agent could be the guinea pig plasmin itself. However, it is more probable that we are dealing with two different agents, since it has been reported that plasmin from different species does not activate either the homologous or bovine plasminogen. This was shown by ASTRUP (1951) and ASTRUP and STERNDOERFF (1952) for bovine plasmin and plasminogen; by LEWIS and FERGUSON (1952) for dog plasmin and plasminogen; and by MÜLLERTZ (1955) for human plasmin and bovine and human plasminogen.

The occurrence of plasmin as well as plasminogen activator can be explained by an effect of peptone on an activator system in the blood leading to the precipitation, with the euglobulin fraction, of free plasminogen activator. The effect of this activator on the plasminogen present in the serum could account for the recorded plasmin activity.

Our results confirm in principle the finding by UNGAR and MIST (1949), that peptone can release fibrinolytic activity in guinea pig serum. We found no evidence in our experiments for

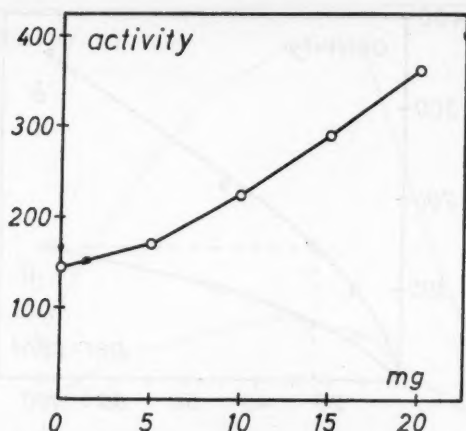


Fig. 3. Varying amounts of bovine plasminogen added to the fibrinolytically active precipitate of a serum-peptone mixture, tested on heated fibrin plates. *Abscissa*: Plasminogen in mg per ml of euglobulin solution. *Ordinate*: Activity.

any significant influence of the incubation period and we observed maximum effect by amounts of peptone 3 to 10 times greater than those reported by UNGAR and MIST.

UNGAR and MIST in their assay method used 15 minutes digestion of a bovine fibrinogen solution, whereas we used a prolonged digestion of a solid fibrin substrate. Since fibrinogen normally contains plasminogen (ASTRUP 1956), it appears likely that the fibrinogenolytic method would respond to plasmin as well as to activators of plasminogen and therefore, in principle, react to the same agents as the standard fibrin plate method. The observed discrepancies could be caused by: (1) differences between the methods with respect to their relative sensitivities to plasminogen activators, to plasmin, or to inhibitory compounds; (2) the occurrence of labile fibrinolytic or inhibitory agents; and (3) differences between the peptones.

The isoelectric precipitation and the composite nature of the precipitates are obstacles to a strictly quantitative estimation of the substances involved in the reaction. The investigation shows, however, that the methods are able to furnish qualitative and semi-quantitative data.

Summary.

1) Peptone, in combination with an isoelectric precipitation, releases fibrinolytic agents in normal guinea pig serum. These results confirm, with a different method, the finding of UNGAR et al.

2) The formation of a plasminogen activator and an active protease is demonstrated.

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The Elicitation of a Drinking Motor Conditioned Reaction by Electrical Stimulation of the Hypothalamic "Drinking Area" in the Goat.

By

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It has been shown that osmotic (ANDERSSON 1952, 1953) and electrical stimulation (ANDERSSON and McCANN 1955 a, b, GREER 1955) of a restricted area in the hypothalamus can cause polydipsia. The fact that destructive lesions involving this same area may result in hypodipsia (STEVENSON 1949) or even adipsia (WITT, KELLER, BATSEL and LYNCH 1952, ANDERSSON and McCANN 1956) gives further evidence that an essential "thirst centre" is located in the hypothalamus. It is possible that the stimulation of this "centre" produces a real sensation of thirst as during such experiments goats move actually towards the proffered water and drink (ANDERSSON and McCANN 1955 a). However, to get a more objective answer to the question whether or not the stimulation of the hypothalamic "drinking area" is connected with the active seeking for water, *i. e.* with the voluntary movements that will guarantee the water supply, the present study was undertaken.

Methods.

Two adult female goats were used for the experiments. At first the drinking of the animals was conditioned in such a way that they were taught to go up the two steps of a staircase placed in the experimental pen to get water. While training was in progress the goats were only

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allowed to drink during the actual training experiments in the pen and hence they were always thirsty when the daily routine started. The experimental pen, the staircase, and the experimenter standing in front of the staircase outside the pen, were the complex conditioned stimulus. The motor conditioned reactions developed during the period of training were such that one goat went up on to the first step with both her forelegs and then put her right hoof on the second step. The other goat climbed the staircase and assumed a standing position on its upper step. Each of these movements was immediately reinforced by giving the animals 100 ml of water to drink and the goats were taught to go down the steps after drinking. These motor reactions were thus elaborated by way of "passive movements" according to the method described by KONORSKI (1948).

The conditioned reaction appeared repeatedly at intervals of about 1 to 2 minutes. The daily experiment consisted of 10 to 15 trials. After about 14 days of training the conditioning of drinking was firmly established. Then, in the same conditioned situation, electrical stimulation within the "drinking area" of the hypothalamus was performed using Hess' technique (1932, 1949). The parameters of stimulation were 0.5—1V and 50 imp./sec. The stimulation experiments and some of the usual training experiments were filmed.

Histological technique: After the animals had been killed by decapitation the heads were perfused with Ringer's solution followed by Bodian's fixative and a block including the hypothalamus was cut out, imbedded in celloidin and cut in transverse sections, 100 microns thick. The sections were stained with toluidin blue to facilitate the localization of the points of stimulation.

Results.

When electrodes had been placed within the "drinking area" of the hypothalamus the previously trained animals were placed in the experimental pen where they could move freely. Some minutes later the usual conditioned reaction appeared and each attempt to find water was reinforced by giving water. After several trials the hitherto thirsty goats were allowed to drink water ad libitum following which the animals ceased completely to perform the learned movement, and refused to drink the water offered. Electrical stimulation of the "drinking area" was then applied, and was found to evoke the conditioned reaction followed by drinking of water. This effect was especially striking in one of the goats in which the effective point of stimulation was situated dorsally in the "drinking area" just lateral to the columna fornicis descendens (Fig. 1). Here the visible effect of a weak stimulation was "pure" polydipsia. The course of this stimulation experiment was the following.

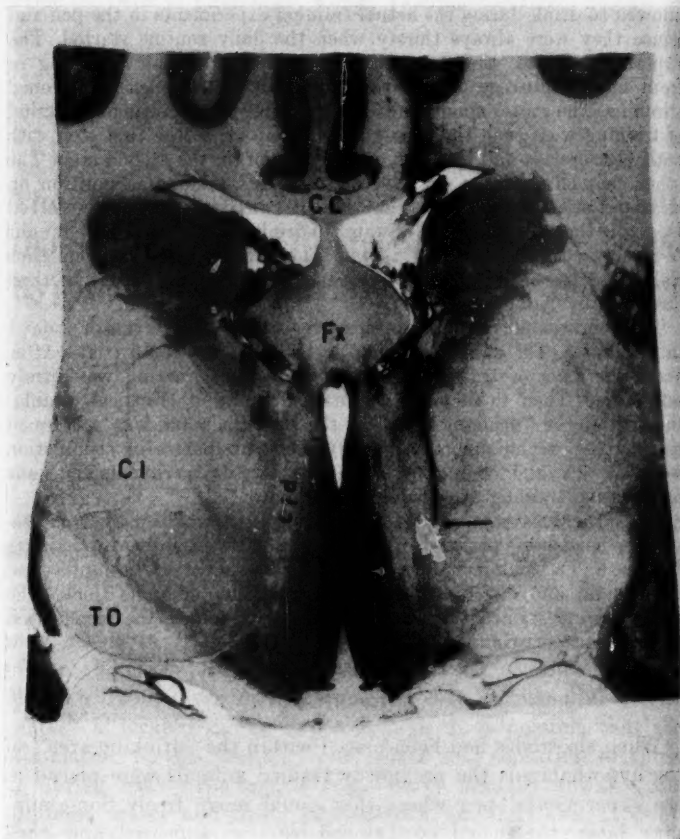


Fig. 1. A transverse section through the anterior hypothalamus of the goat in which the drinking motor conditioned reaction was most efficiently elicited by electrical stimulation within the hypothalamic "drinking centre". The section is directed along the track of the effective electrode. The point of stimulation is indicated by the arrow.

C. C. : corpus callosum

Cd : N. Caudatus

c.f.d. : columna fornix descendens

C. I. : capsula interna

Fx : fornix

P. V. : N. paraventricularis

S. O. : N. supraopticus

T. O. : tractus opticus

Within 1 to 3 secs of the onset of stimulation (0.5 V), the goat went up the first step of the staircase and put her right foreleg on to the second step stamping impatiently on it with her hoof. When water then was offered she drank it in large gulps. As soon as the period of stimulation ended the goat suddenly stopped

drinking, took her right leg off the upper step and went down on the floor of the pen. She thus exhibited exactly the same motor conditioned reaction as previously displayed during the period of training. Although the stimulation was repeated more than 20 times, at intervals from 10 secs to several minutes, it never failed to elicit the characteristic reaction. However, when in consequence of drinking during many periods of stimulation the goat gradually became overloaded with water, the latency of the conditioned reaction increased and the amount of water drunk during each period of stimulation became smaller. At this time an increase of the strength of stimulation to 1V caused the reaction to grow stronger again. The amount of water drunk during the experiment was 6.5 liters. In the intervals between the stimulations the goat refused to drink the water offered and never performed the learned movements. Stimulation of other points in the diencephalon outside the "drinking area" did not elicit the conditioned reaction.

Discussion.

The results obtained show that the electrical stimulation of the hypothalamic "drinking centre" can elicit the motor conditioned reaction connected with drinking. In other words, the pattern of motor excitation seemingly established in the cerebral cortex as a result of previous training may be reproduced as a consequence of the stimulation of this "centre", which, thus, in terms of higher nervous activity, may be considered the origin of an unconditioned drinking reflex. This is in accordance with the suggestion, made by WYRWICKA (1952), on the basis of studies of alimentary motor conditioned reflexes, that the path of a motor conditioned reaction must run through the centre of the unconditioned reflex.

Some analogous data were reported by GRASYÁN, LISSÁK and KÉKESI (1956) who found that an alimentary motor conditioned reflex could be elicited by the stimulation of some parts of the hypothalamus in cats. They interpreted this phenomenon as a non-specific facilitation of conditioned reflexes caused by the hypothalamic stimulation. As, however, it is possible that their stimulations involved an activation of the hypothalamic "feeding centre" (BRÜGGER 1943, ANAND and BROBECK 1951, DELGADO and ANAND 1953, LARSSON 1954) their results may also be interpreted in the above mentioned way.

Summary.

When a drinking motor conditioned reaction was firmly established in goats it was possible to elicit the same reaction by electrical stimulation of the hypothalamic "drinking centre".

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Action Potential and Mechanical Response of Isolated Cross Striated Frog Muscle Fibres at Different Degrees of Stretch.

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The excitable membrane which surrounds each muscle fibre receives its stimulus from the nerve and transmits it to the ends of the muscle fibre. This function is associated with the propagated change in membrane potential which precedes the mechanical response (BERNSTEIN 1871, LUCAS 1909). The correlation between the membrane changes and the mechanical response is however far from clarified. Recent evidence indicates that a local depolarisation of the surface membrane is connected with the initiation of contraction (KUFFLER 1946, KATZ 1950, ROTSCHUH 1956). On the other hand, there are many examples of localized contractions without a propagated action potential (GELFAN and BISHOP 1933).

It is the aim of the study presented here to investigate certain relationships between action potential and mechanical response in the isolated muscle fibres: (1) the time relationship between the action potential and the mechanical response; (2) the changes in the conduction velocity of the action potential and in the mechanical response when the fibre is transferred from Ringer's solution to moist air; and (3) the changes in action potential amplitude and propagation velocity associated with the decrease in the mechanical response as obtained by increasing the degree of stretch.

The changes in propagation velocity induced in *individual* fibres by variation of their diameter through stretch are discussed with regard to their bearing on the membrane resistance and capacitance and are compared with the rectilinear relationship found previously for fibres of different diameters (HÅKANSSON 1956).

Method.

Fibres from the frog's semitendinosus (*Rana temporaria*) were isolated as described (HÅKANSSON 1956). They were kept in Ringer's solution bubbled continuously with a gas mixture containing 1 per cent CO_2 and 99 per cent O_2 (pH 7.2–7.4). The Ringer's solution contained per liter 6.7 g NaCl, 0.2 g KCl, 0.4 g CaCl_2 (anhydrous), 0.4 g glucose, 30 g dextrane and 1.6 ml (2.5 per cent) NaHCO_3 .

Recording of the action potentials.

For recording of the *extracellular* action potential two glass capillary electrodes were used containing a platinum wire inserted to within 50 μ from the tip and filled with Ringer's solution. They had an opening of 30 to 50 μ and were placed at a distance of 38 μ from the surface of the fibre. The distance between the stimulating electrode and the recording electrode nearest to it was at least 4 mm, and between the two recording electrodes about 4 mm. When determining conduction velocity the two electrodes were connected to the input of the same amplifier and a platinum electrode in the Ringer's solution was used as common reference. The action potentials from the two recording points were thus recorded on the same beam of the oscilloscope and the conduction velocity was calculated from the time interval between them and from the distance between the corresponding leading-off electrodes. When measuring action potential amplitude only one electrode was coupled to the input of the amplifier.

The method of stimulation, recording of the extracellular and intracellular action potentials, and the arrangements for prevention of the stimulus escape have been described as has the method of recording in air (HÅKANSSON 1956, 1957).

Recording of isometric twitch tension.

The one tendon end of the muscle fibre situated within the stimulus isolation chamber (Fig. 1) was fixed to a movable glass rod by which the length of the fibre could be varied within wide limits. The other tendon end of the fibre was connected to a mechano-electronic transducer (RCA 5734) for recording of isometric twitch tension (CURTIS 1950, TALBOT, LILIENTHAL, BESER and REYNOLDS 1951, HILL 1951). To this purpose the tendon end was fixed to a seventy mm long glass capillary tube (external diameter 0.2–0.3 mm) by means of a nylon

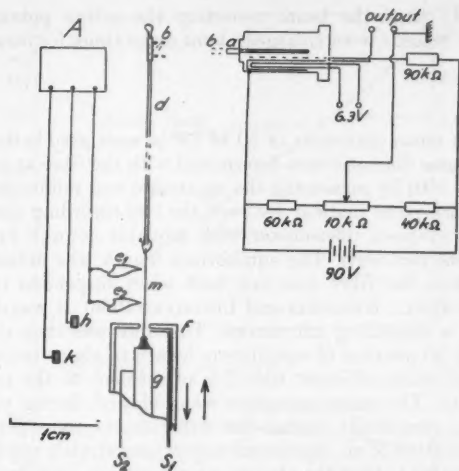


Fig. 1. Arrangement for the simultaneous recording of the action potential and the isometric tension of a frog muscle fibre in Ringer's solution.

- (a) Anodal pin of the mechano-electronic lower tendon end of the fibre is attached.
 (b) Brass rod soldered on (a).
 (c) Glass tube fixed on (b).
 (d) Glass tube connecting the cantilever and the upper tendon end of the fibre.
 (e₁), (e₂) recording electrodes.
 (f) Stimulus isolation chamber.
 (g) Movable perspex pin to which the
- (h) Indifferent electrode.
 (k) Earth electrode.
 (S₁), (S₂) Stimulating electrodes. (S₁ = cathode)
 (m) Isolated muscle fibre.
 (A) Amplifier for recording action potentials.

Right: Wiring diagram for the transducer.

thread, and the other end of the glass capillary was attached via a metal rod to the movable anode of the transducer. The metal rod passed through a hole which restricted its total movement to 1° . To reduce drift and noise the transducer tube was surrounded by a series of vertical metal radiator plates for heat dissipation. The tube and its cathode resistor were two branches of a bridge circuit (Fig. 1) and the moving arm of the balancing potentiometer (P) was connected to earth. The signal across the cathode resistor was via a d. c. amplifier connected to the one beam of a double beam oscilloscope (DuMont 279). Calibration of the transducer including connecting glass tube and metal rod gave a signal from the transducer which was proportional to the force applied up to at least 250 mg. The resonance frequency of the transducer with its glass rod was 800 c. p. s. for longitudinal vibrations of the glass rod. Its damping was small. In addition the glass rod itself could vibrate with a transverse frequency of about 50 c. p. s. This did not cause any appreciable distortion of the recording of the initial twitch tension. To record the entire course of contraction the beam of the dual beam oscilloscope connected to the transducer operated at a slower

sweep velocity than the beam recording the action potentials. The photographic records were enlarged about seven times for measurement.

Procedure.

Fibres with mean diameters of 50 to 135 μ were used in these experiments. The mean diameter was determined with the fibre at equilibrium length ($L_e = 100$) by measuring the maximum and minimum diameter of the fibre at a point midway between the two recording electrodes by means of an eyepiece micrometer with movable cobweb (uncertainty less than three per cent). The equilibrium length was defined as that length at which the fibre was just taut when suspended in Ringer's solution (BUCHTHAL, KNAPPEIS and LINDHARD 1936). It was determined by means of a measuring microscope. The fibre was then stretched in steps of 15 to 20 per cent of equilibrium length to about twice equilibrium length allowing sufficient time for adjustment to the new tension at each length. The same procedure was followed during stepwise release. Unless specifically stated the experiments were performed at 20° C. The duration of an experiment comprising stretch and release was about 30 minutes so that the changes in conduction velocity and action potential amplitude with time after isolation were only small; they were however corrected for as described (HÅKANSSON 1956).

Uncertainty of measurements.

The conduction velocity of the action potential was determined with an uncertainty of less than three per cent. When considering the changes in the amplitude of the extracellular action potential with stretch of the fibre it must be taken into account that the change in geometry of the fibre by itself induces changes in the amplitude. These changes depend on the diameter of the fibre and may be estimated in the following way: During stretch the distance of the electrode from the fibre was kept constant at 38 μ at different degrees of stretch. Therefore, with increasing stretch the distance to the fibre axis decreased causing an increase in action potential amplitude (HÅKANSSON 1957). At 100 per cent stretch this decrease in distance caused an increase in action potential amplitude of 25 per cent in the case of the largest fibres (135 μ diameter), of 12 per cent for fibres of intermediate diameter (100 μ), and of 7 per cent for the smallest fibres examined (50 μ).

The action potential amplitude depends on the ratio between electrode opening and fibre diameter (HÅKANSSON 1956). The electrode opening is small as compared with the fibre diameter of the largest fibres at all degrees of stretch. In the smallest fibres, fibre diameter and electrode opening were of the same order of magnitude at all degrees of stretch. Therefore, in these instances stretch did not influence the effect of the size of the electrode opening on action potential amplitude. However, in the case of fibres with an intermediate diameter, for example 90 μ , the fibre diameter was about twice the size of the electrode opening at equilibrium length, while at 100 per cent stretch the diameters were

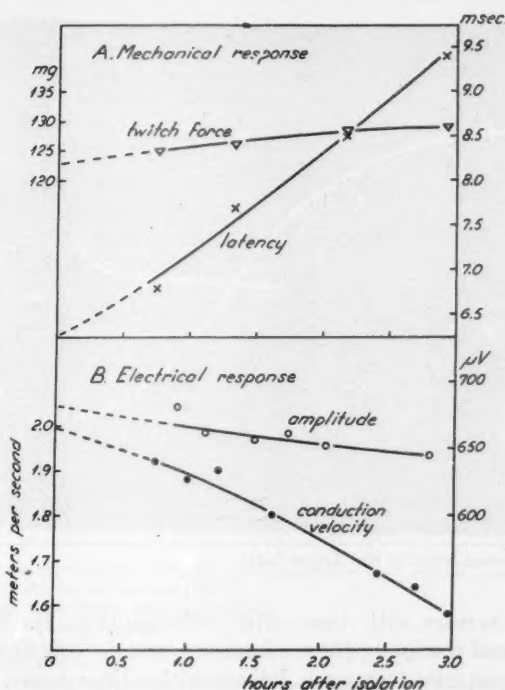


Fig. 2. Mechanical response and latency (A), action potential amplitude and conduction velocity (B) as a function of time after isolation of the muscle fibre (20° C). Left ordinate: Twitch force (A) and conduction velocity (B). Right ordinate: Twitch latency (A) and action potential amplitude (B). The scales are such that equal relative variations are identical on the graphs.

only slightly different. This resulted in a decrease in amplitude of five to ten per cent at 100 per cent of stretch.

The resulting change in amplitude to be expected from the change in distance between electrode and fibre axis with stretch was therefore an increase of 25 per cent in the case of the largest fibres and of 5 per cent of fibres of intermediate and small diameter.

Results.

1. *Action potential and mechanical response.*
 - a) *Constancy of the response.*

Unlike the conduction velocity and amplitude of the action potential, the twitch tension showed no decrement but rather

Table 1.

Conduction velocity of the action potential and mechanical response with the fibre in Ringer's solution and in moist air.

Fibre diameter μ	Temperature $^{\circ}\text{C}$	Conduction velocity of the action potential		Twitch tension		Mechanical response latency		Maximum rate of tension rise	
		Ringer m per sec	Air m per sec	Ringer mg	Air mg	Ringer msec	Air msec	Ringer mg per msec	Air mg per msec
126	20—22	2.37	1.19	153	125	10	15	7.0	5.3
105	10	1.29	0.72	146	131	25	30	4.0	5.3
98	17	1.39	—	143	112	11	16	9.7	5.6
95	18	1.08 ¹	—	117	105	13	15	5.3	4.0
94	8	0.50	0.23	161	102	30	60	6.2	3.0
84	10	1.20	—	165	122	25	35	4.8	3.3
100	14	1.31	0.71	148	116	19	29	6.2	4.1
Change in per cent from Ringer's solution to air			—46		—21		+52		—29

¹ after several hours in the Ringer bath.

a slight increase with time after isolation (Fig. 2). The only pronounced change in the mechanical response with time was an about three msec increase in latency within three hours, from 6.5 to 9.5 msec (20°C).

b) *The responses recorded in air and in Ringer's solution.*

The increase in external resistance when the muscle fibre was transferred from Ringer's solution to humid air was associated with changes both in the action potential (HÅKANSSON 1957) and in the mechanical response. The conduction velocity of the action potential decreased by about fifty per cent. The latency of the mechanical response increased likewise by fifty per cent. The twitch tension decreased by twenty per cent and its rate of rise by thirty per cent (Table 1).

c) *Time relationship between action potential and mechanical response.*

Simultaneous recording of the extracellular action potential and of twitch tension demonstrated that the extracellular potential

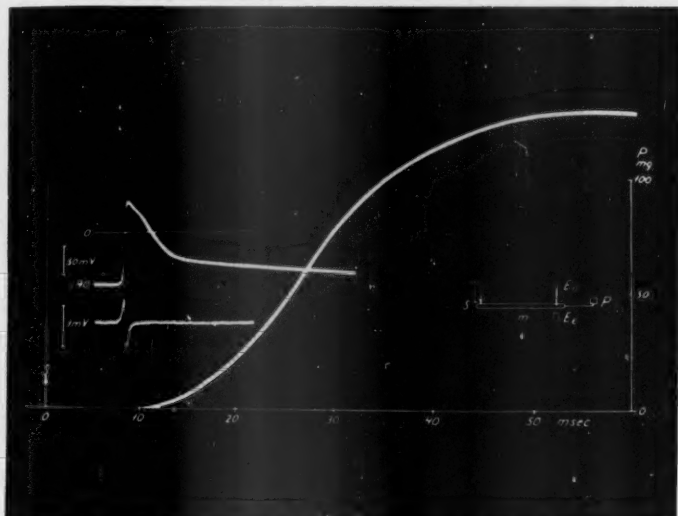


Fig. 3. Time relationships between the intracellular and extracellular action potential and the twitch tension (19°C).

Left above: intracellular action potential.

Left below: action potential.

Right: Rising phase of twitch tension (right ordinate, tension in mg).

S: Time for stimulus.

The inset to the right gives the position of the stimulating and recording electrodes. The extracellular action potential and the twitch tension were recorded simultaneously on the two beams of the oscilloscope. The fibre was about 20 per cent stretched, it had a length of 11 mm and a mean diameter of $110\ \mu$. The conduction velocity of the action potential was 1.5 m per sec.

Regarding the time relationship between the extracellular and the intracellular action potential see HÅKANSSON (1957).

and hence the rising phase of the intracellular potential (HÅKANSSON 1957) occurred within the latency period of the mechanical response (Fig. 3).

In these experiments the fibres were stimulated at the one end and the action potential recorded at the other end of the fibre (Inset, Fig. 4). Therefore, the latency of the electrical response included the time necessary for propagation of the action potential from the stimulating electrode (cathode) to the leading-off electrodes (7–8 msec, 20°C) and the rising phase of the propagated disturbance of the membrane had traversed nearly the entire length of the fibre before the first sign of positive twitch

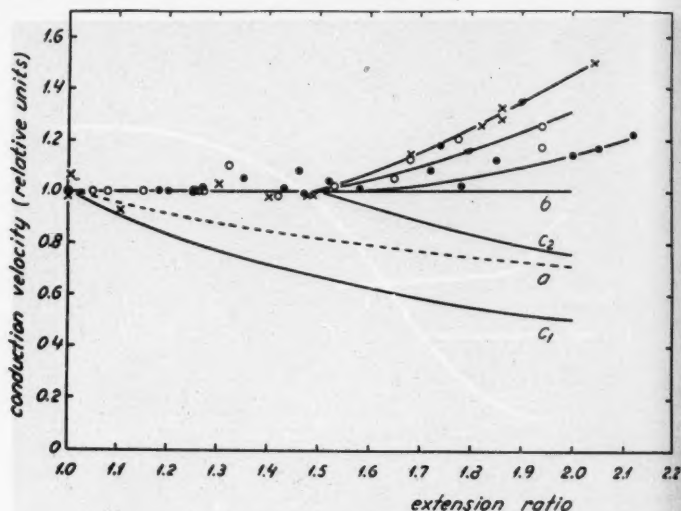


Fig. 4. Conduction velocity of the action potential as a function of stretch in isolated fibres of different diameter.

- × fibres with a mean diameter above 125μ
- fibres with a mean diameter of $95-125 \mu$
- fibres with a mean diameter below 95μ

Ordinate: conduction velocity in units of equilibrium length.

Curves a — c are theoretical relationships:

- a) assuming proportionality between mean diameter and conduction velocity (HÅKANSSON 1956)
- b) "folded membrane" (MARTIN 1954)
- c₁) "unfolded membrane" according to MARTIN (1954)
- c₂) a "folded membrane" assumed to unfold at 50 per cent stretch.

tension appeared (Fig. 3). Corrected for the time required for conduction of the action potential from the point of stimulation to the leading-off electrode, the extracellular action potential terminated before the *middle* of the mechanical latency. Thus, the extracellular action potential terminated even before the onset of the small drop in tension, the latency relaxation, occurring before tension development starts (SANDOW 1944, ABBOTT and RITCHIE 1951, MAURO 1951, HILL 1951). The intracellular action potential, on the other hand, lasts so long that it attains its resting value at about the peak of isometric twitch tension. Provided that slack is eliminated by a slight stretch of the fibre, the mechanical response arising at the stimulating cathode manifests itself instantaneously by an increase in fibre tension due to the

Fig.
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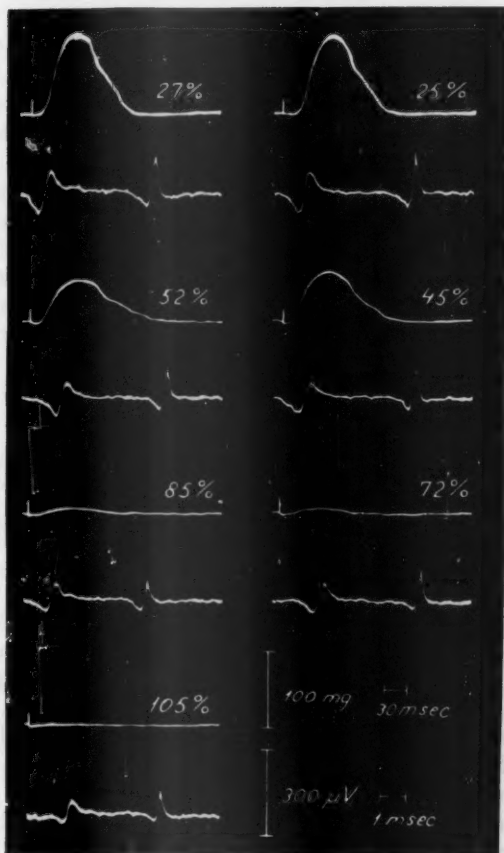


Fig. 5. Action potentials and twitch tension of the isolated muscle fibre at different degrees of stretch (20°C). The upper beam of the oscilloscope recorded twitch tension, the lower beam, at a higher speed, the action potential from two points of the fibre 5.5 mm apart. The moment of stimulation is therefore only visible on the twitch tension records.

Left column (from above) stepwise increase in length.

Right column (from below) stepwise release. The figures above the tension record indicate the stretch in per cent of equilibrium length. The length of the fibre was 11 mm, its mean diameter $80\text{ }\mu$.

stretch of the series elastic element. The higher the rate of tension rises, the earlier tension will appear. Therefore, the mechanical latency is slightly longer when the stimulus is applied to one end

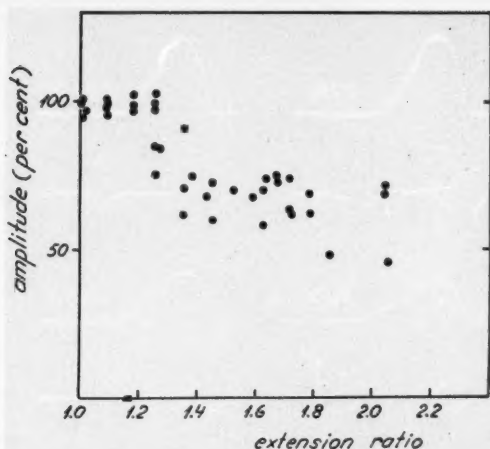


Fig. 6. Amplitude of the extracellular action potential as a function of stretch (20° C). Ordinate: amplitude in per cent of its value with the fibre at equilibrium length. Abscissa: fibre length in units of equilibrium length.

of the muscle fibre than when the stimulus is applied to the nerve or in a way that a larger portion of the fibre is activated simultaneously.

2. *The effect of stretch on conduction velocity, potential amplitude and twitch tension.*

a) *Conduction velocity of the action potential.*

As a function of stretch the conduction velocity remained nearly unaltered up to a stretch of fifty or sixty per cent (Fig. 4). With a further elongation the velocity increased, the increase being the greater the larger the initial fibre diameter: the maximal increase in velocity at 100 per cent of stretch was 14 per cent for small, 30 per cent for intermediate and 50 per cent for large fibres.

The course of a typical experiment is illustrated in Fig. 5. The left column shows the action potentials during stretch recorded from two points about 5.5 mm apart and the simultaneously recorded mechanical response. The time interval between the two potentials indicates the conduction velocity; at 27 and 52 per cent of stretch the velocity was 1.27 and 1.31 m per sec respectively, identical with the velocity over the fibre at equilibrium length

(1.26 m per sec). At 85 and 105 per cent elongation the conduction velocity was 1.42 and 1.47 m per sec respectively. Thus, with stretch exceeding 52 per cent the conduction velocity showed a clear increase.

At release (right column, Fig. 5), the conduction velocity decreased again being 1.37 m per sec at 72 per cent of stretch and attained the initial value of 1.27 m per sec at 43 per cent of stretch.

The increase in conduction velocity at stretch exceeding fifty per cent occurred immediately after the stretch, even before the fibre had mechanically adjusted to the new length. During the stepwise release the conduction velocity attained the reduced value with a delay, the final value at a given length first being reached after three or four twitches evoked at fifteen to sixty second intervals.

b) *Action potential amplitude.*

In a previous study it was shown that the amplitude of the extracellular action potential of different muscle fibres increased with increasing fibre diameter (HÅKANSSON 1956). Did a similar relation apply to the individual fibre when changing its diameter the action potential amplitude would decrease with increasing stretch. This was actually found in the experiments, the reduction being 25 to 30 per cent at 50 per cent of stretch and 40 per cent at 100 per cent of stretch (Fig. 5 and Fig. 6). This decrease was found not to depend on the initial fibre diameter.

c) *The mechanical response.*

In agreement with the findings of previous investigations (*e. g.* ASMUSSEN 1936) the twitch tension of the isolated muscle fibre decreased with increasing stretch, *i. e.* with increasing resting force. The decrease was disproportionately large as compared with the increase in conduction velocity or with the decrease in action potential amplitude (Fig. 5). This was most evident at a stretch of 100 per cent, *i. e.* a length at which the resting force exceeds the maximum twitch force. Here a mechanical response was barely detectable while the conduction velocity was increased by 30 per cent and the action potential amplitude reduced by 40 per cent. That this is no irreversible block of the mechanical response can be seen from the reappearance of mechani-

cal tension at release, the initial values of twitch tension, conduction velocity and action potential amplitude being regained. In all instances stepwise stretch and release could be repeated several times with a high degree of reversibility for the quantities measured.

Discussion.

The findings concerning the time relationship between action potential and mechanical response in the isolated muscle fibre confirm results on whole muscle (BERNSTEIN 1871, LUCAS 1909) though without the uncertainty which derives from the presence of many fibres of different lengths (BUCHTHAL, KAISER and ROSENFALCK 1951). The extracellular action potential invariably precedes the mechanical response. In the isolated fibre the interval between the onset of electrical response and of tension development is about 10 msec with both stimulating electrodes placed at one end of the fibre (20° C). The phase of repolarization of the intracellular action potential on the other hand outlasts the mechanical latency period and the resting internal potential is first attained at about the peak of isometric tension (20° C) (HOROWITZ and HODGKIN 1957).

Previous findings regarding the change in conduction velocity with stretch are divergent: In whole muscle, MARTIN (1954) recently confirmed HOFFMAN's (1912) experiments which suggested a slight or no change in conduction velocity. Leading-off from isolated fibres within the abdominal muscle of frog, WILSKA and VARJORANTA's (1939) experiments indicated a rectilinear relationship between conduction velocity and stretch in the range of 0 to 90 per cent stretch with an increase of 10 per cent per fifty per cent stretch. In giant nerve fibre of *Lumbricus* and Loligo, BULLOCK, COHEN and FAULSTICK (1950) did not find any changes of conduction velocity with stretch. However no data are given as to the amount of stretch applied. RUD¹ (1957) in frog's sciatic nerve found no change up to 20 per cent of stretch. With further stretch by a few per cent up to rupture the conduction velocity decreased by 10 per cent.

In whole muscle with fibres of widely varying lengths interpretation of findings may be obscured in that at a given extension of the muscle the stretch of the individual muscle fibres differs

¹ Personal communication.

widely (BUCHTHAL, KAISER and ROSENFALCK 1951). Keeping this in mind the findings of MARTIN (1954) seem compatible with the relationship presented here since total muscle can not be stretched to the same extent as the isolated fibre¹. Hence, it might very well be that the stretches applied by MARTIN only slightly exceeded the range within which the conduction velocity was constant in the isolated fibre.

In different unstretched fibres conduction velocity varied proportionally with circumference (HÅKANSSON 1956). Did a similar relationship apply to the individual fibre when its diameter is changed by stretch, the velocity would be inversely proportional to the square root of the relative fibre length (curve a, Fig. 4). This is at variance with the experimental findings the actual change being an increase and not a decrease.

In terms of the cable theory the conduction velocity (V) depends on the transverse membrane resistance per cm² R_m , the membrane capacitance per cm² C_m , the specific internal resistance of the fibre R_i , the fibre radius at equilibrium length r_0 , and the fibre length $\frac{L}{L_0}$:

$$V = \frac{S}{\sqrt{2R_m}} \cdot \frac{\sqrt{r_0}}{C_m \sqrt{R_i}} \cdot \sqrt[4]{\frac{L_0}{L}} \quad \dots\dots\dots (1)$$

where S is the safety factor (RUSHTON 1937, OFFNER, WEINBERG and YOUNG 1940, KATZ 1948) and disregarding the external longitudinal resistance. The factor containing fibre length $\frac{L}{L_0}$:

implies a decrease in conduction velocity with increasing stretch. The increase in velocity which in fact was found in the present experiments could arise from an increase in the safety factor S and from a decrease in specific membrane resistance (R_m) and capacitance (C_m), or from both. It seems reasonable to assume that the specific internal resistance (R_i) is not altered by stretch. The safety factor S may be expressed as:

$$S = \frac{E_0}{2E_1 R} - 1 \quad (\text{KATZ 1939})$$

¹ MARTIN (1954) expresses the length in units of the muscle length in situ with the leg fully extended, and equilibrium length used as unit length in the present experiments corresponds to 65 per cent of the unit length used by MARTIN.

where E is the resting membrane potential and E_1 the critical potential change required for local excitation while R and ρ are components of the membrane resistance. Hence an increase in the safety factor implies an increase in the ratio between resting potential and critical potential or a relative decrease in the purely ohmic component of the membrane resistance, or both. Therefore the present experiments suggest changes in specific membrane resistance and capacitance with stretch but it cannot be decided whether the changes are confined to only one of these membrane characteristics.

That mechanical deformation influences the stability of the membrane is indicated by the increase in spontaneous activity of calcium deficient skeletal muscle with stretch (BÜLBRING, HOLMAN and LÜLLMANN 1956).

To account for the high capacitance of the muscle fibre membrane KATZ (1949) has suggested the possibility of a folded structure and MARTIN (1954) explains the independence of conduction velocity from stretch in terms of this model (Fig 4, curve b). For an unfolded membrane MARTIN (1954) predicts a decrease in conduction velocity with stretch assuming the membrane capacitance to vary in inverse proportion to the thickness of the membrane and the membrane resistance to be proportional to the surface area of the fibre or to the membrane thickness (Fig. 4, curve c_1 , c_2). While a conduction velocity in the isolated fibre which is constant up to a stretch of fifty per cent is consistent with the concept of a folded membrane the increase in velocity with further elongation requires more complicated assumptions than those suggested by MARTIN (1954) in the case of an unfolded membrane. This increase was not due to any damaging effect of the higher degrees of stretch since it was completely reversible and there was no indication of a change in threshold for the electrical and the mechanical response (BUCHTHAL 1942).

In whole muscle the amplitude of the action potential increases by about 25 per cent with stretch from length 67 to length 122 (expressed in per cent of muscle length in situ). This increase has been attributed to the reduction with stretch in the cross sectional area over which the action currents spread (MARTIN 1954). With the isolated muscle fibre placed in Ringer's solution there was a 40 per cent decrease in the amplitude of the extracellularly recorded action potential with stretch from length 100 to 200, expressed in per cent of equilibrium length. For the nerve action

potential LORENTE DE NÓ (1947) has deduced a proportionality between action potential amplitude and cross sectional area of the potential source. In previous experiments this was shown to apply to isolated muscle fibres of different diameters (HÅKANSSON 1957). An elongation of 100 per cent being associated with a fifty per cent decrease in cross sectional area would in turn cause the externally recorded action potential amplitude to decrease by 50 per cent. This agrees within the measuring accuracy with the 40 per cent decrease in amplitude found in the experiments.

Under normal conditions the process of excitation as manifested by the propagated action potential must be assumed to activate the contractile mechanism. The interrelation between excitation and contraction is, however, still obscure. The present findings indicate the lack of any proportionality between the amplitude of the propagated action potential and the mechanical tension developed during contraction. At an elongation at which the mechanical response could no longer be elicited the amplitude of the extracellular action potential was reduced by no more than forty per cent and this reduction is readily explicable in terms of the spread of the volume conducted action potential. It might be argued that the lack of any tension development under these circumstances is due to the marked deformation of the contractile elements of the fibre. However, recent experiments with the fibre in hypertonic sodium Ringer's, where this objection does not apply, have given similar results (HODGKIN and HOROWICZ 1957). Conversely, I have found that the mechanical response still retained its original magnitude under such conditions that the action potential amplitude and its conduction velocity were greatly reduced, for example five or six hours after isolation of the muscle fibre.

Similarly, when the conduction velocity was halved by changes in the surrounding medium, *e. g.* by placing the muscle fibre in moist air, there was a 50 per cent lengthening of the latency period and an only slight (20 per cent) decrease of the tension developed in the twitch. The increase in latency is a reflection of the lower conduction velocity. The decrease in twitch tension might be described by a faster decrease in "active state" (HILL 1949) with the fibre in air as compared with Ringer's solution. Supposing the maintenance of the active state to depend on a certain degree of depolarization the faster decrease in air might be attributable to the shorter duration and wave length of the

action potential in air (HÅKANSSON 1957). In any case, the reduction in twitch tension is hardly due to the decrease in conduction velocity: There was no decrement with time in the twitch tension in spite of a reduction in conduction velocity.

Summary.

1) Simultaneous recording of the action potential and the mechanical response showed that the rising phase of the intracellular action potential had traversed the entire length of the fibre before the first sign of twitch tension appeared. Correcting for the time necessary for propagation of the action potential to the leading-off electrode the extracellular action potential terminated before the middle of the mechanical latency (Fig. 3).

2) As a function of stretch the conduction velocity of the action potential remained unaltered up to a stretch of about fifty per cent. With further elongation it increased, the increase being the greater the larger the fibre diameter. The maximum increase in velocity was fifty per cent with 100 per cent stretch in fibres of $135\ \mu$ diameter (Fig. 4). The increase in conduction velocity could not be accounted for by assuming a "folded membrane". It is explained in terms of a change in the specific resistance and capacitance of the muscle fibre membrane with stretch.

3) The decrease in amplitude of the action potential with stretch amounting to 40 per cent at 100 per cent stretch is attributable to the decrease in the cross sectional area of the muscle fibre (Fig. 6).

4) There was no correlation between the decrease in twitch tension and the changes in the electrical response with stretch. At 100 per cent stretch where twitch tension was practically zero the amplitude of the extracellular action potential was reduced by only forty per cent (Fig. 5).

5) The 50 per cent decrease in conduction velocity when transferring the muscle fibre to humid air was associated with a decrease in twitch tension of 20 per cent, with an increase in the mechanical latency of about 50 per cent and a decrease in the maximum rate of rise of the twitch tension of 30 per cent. The shorter duration and wave length of the action potential with the fibre in air is suggested to be the cause of the changes in the mechanical response (Table 1).

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Biogenesis of Histamine Studied by Its Distribution and Urinary Excretion in Germ Free Reared and Not Germ Free Rats Fed a Histamine Free Diet.

By

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It is generally agreed that the histamine excreted in the urine is derived in part from exogenous and in part from endogenous sources. The first part originates from histamine in the food and histamine formed by bacterial decarboxylation of histidine in the intestine, the other part from histamine formed by mammalian histidine decarboxylase. Furthermore, the urinary histamine of endogenous origin has two possible sources: 1) histamine released from tissue cells in the course of normal histamine turnover and liberation, 2) histamine formed by mammalian histidine decarboxylase and excreted directly without having been retained in the tissues, principally histamine formed in the kidneys.

The origin of the histamine contained in the various tissues of the body is less clear. Because of the failure to demonstrate histidine decarboxylase in the tissues of some species in vitro, such as man, dog and cat (WATON 1956), it is thought by some investigators that the histamine in mammalian tissues originates from histamine absorbed from the intestine where it is formed by bacteria. This view is supported by the experiments of WILSON (1954), who reports that inhibition of the intestinal bacterial flora in rats by orally administered antibiotics causes a decrease in the urinary excretion of histamine to values of about half of nor-

mal and in addition a significant reduction in the histamine content of the wall of the small intestine. The bearing of the experiments of WATON and WILSON has been fully discussed by GAD-DUM (1956).

Experiments by approaches and techniques different from those mentioned above suggest that all the histamine stored in tissues is produced independently of exogenous sources. It was noted in cats that complete withdrawal of food for as long as seven days did not significantly alter the content of histamine in the gastrointestinal mucosa (HAEGER and KAHLSON 1952). It was further demonstrated in cats that the concentration of histamine in the stomach wall is uniform in the mother and her foetuses, whereas after birth the young go their own way and establish individual levels of histamine, indicating some sort of regulatory mechanism operating via the blood stream and controlling the level of histamine in the gastrointestinal tract (HAEGER, KAHLSON and WESTLING 1953). These observations are not compatible with the notion of a tissue "bag" into which any molecule of histamine, endogenously or exogenously produced, may enter.

More direct evidence concerning the biogenesis of histamine has been provided by SCHAYER and his colleagues in experiments with C^{14} -histidine and C^{14} -histamine. SCHAYER (1952) found that C^{14} -histamine could not be detected in the organs of guinea-pigs after injection with radioactive histamine, while after injection with radioactive histidine, C^{14} -histamine could be detected in the tissues and urine. Even histamine-depleted rats did not bind exogenous C^{14} -histamine (SCHAYER and SMILEY 1954). Suspensions of rat mast cells decarboxylate C^{14} -histidine and retain the resulting histamine, whereas exogenous histamine is not retained in the mast cells (SCHAYER 1956). With SCHAYER's approach it was never possible to demonstrate binding of exogenous histamine in intact animals. SCHAYER's observations and calculations bring him to the notion of a "limited number of binding sites" holding one molecule of histidine, which can get in as there is no block, and which is then decarboxylated and retained as histamine in the same site (SCHAYER 1956).

The ingenious experiments of SCHAYER and his collaborators illuminate the biogenesis and incorporation of extremely small amounts of injected radioactive compounds during relatively short periods of observation. It was thought useful to supplement these experiments by studies of the all over biogenesis and urinary

Table 1.
Diet D5.

Casein	22	%
Wheat starch	63	%
Arachis oil	10	%
Salt mixture HMW ¹	4	%
Vitamin mixtures	1	%
Vitamins added per 100 g diet:		
Vitamin A	2 100	IU
Vitamin D	450	IU
Vitamin E	50	mg
Vitamin K	10	mg
Thiamine	5	mg
Riboflavin	2	mg
Pyridoxin	2	mg
Calcium pantothenate	10	mg
Nicotinamide	20	mg
Choline	200	mg
Inositol	100	mg
P-Aminobenzoic acid	30	mg
Biotin	0.1	mg
Folic acid	2	mg
Vitamin B ₁₂	0.002	mg
Ascorbic acid	100	mg

¹ According to HUBBELL, MENDEL and WAKEMAN (1937).

excretion of histamine under conditions where any interference by exogenous histamine can be strictly excluded. This object is achieved in germ free reared rats raised and maintained on a histamine free diet. In the present study observations on germ free reared rats are brought in relation to corresponding observations made on a group of not germ free rats fed the same histamine free diet.

Methods.

Rearing and care of germ free rats. The germ free animals were reared as described by GUSTAFSSON (1948), with slight modifications of the rearing apparatuses, which will be reported elsewhere. After weaning at 20–22 days, the rats were fed diet D5 and water ad lib. (Table 1.) The diet was mixed with 50 % water and autoclaved at 121° C for 20 minutes. This diet of which adult rats eat about 15 g daily is histamine free ($< 0.0024 \mu\text{g/g}$) as judged from extraction and boiling with hydrochloric acid and estimates on the guinea-pig's ileum. The rats were kept in metabolism cages, and faeces and urine were collected from individual rats in 24 hours samples. To prevent interference by bacterial growth in the urine from not germ free rats the collecting vessels contained 0.2 ml 10N HCl, which brought the pH of the collected urine to between 1 and 2. In control tests there was good agreement between

the content of free histamine in freshly passed urine and in the same urine stored for 48 hours with added HCl. The hydrochloric acid was added to the urine samples from the germ free animals after the transfer from the apparatus.

Since the autoclaving of the diet could cause changes in its composition, rats were investigated from the three groups ordinarily kept in the germ free studies.

1. Germ free rats on autoclaved diet D5.
2. Not germ free rats on autoclaved diet D5.
3. Not germ free rats on not autoclaved diet D5.

The germ free as well as the not germ free rats were offsprings of a male and female from a strain of rats reared for many years at the Institute of Histology. Both handfed rats of the first generation and animals of the second generation born and reared by germ free mothers were studied.

Sterility tests were performed twice a week on faeces and waste from the germ free apparatuses. The following culture media were used under aerobic and anaerobic conditions: N. I. H. thioglycollate broth, fluid thioglycollate medium, brain-liver-heart medium and Sabouraud's agar. Direct microscopy on faecal smears was also performed. At least once a month sterility tests were independently carried out by members of the staff of the Department of Bacteriology.

Mast cells. In preliminary studies where the number of mast cells were counted in the mesentery of germ free and not germ free rats no differences were noted.

Extraction of tissues for histamine. The rats were killed by a blow on the head and exsanguinated from the cut carotid arteries. The various samples were removed, dried between filter paper, weighed, minced with scissors, and ground with sand under addition of 10% (w/v) trichloroacetic acid. After standing for 3 hr or longer, the precipitates were filtered off and the filtrates treated according to CODE's (1937) modification of the method of BARSOUM and GADDUM (1935).

Whole rats were extracted with the exclusion of the bony parts. The skin was removed and the soft tissues removed from the bones which were discarded. The pooled tissues were minced by means of a Waring blender in a suitable volume of trichloroacetic acid. The filtrate was treated as mentioned above and aliquots assayed for histamine.

Preparation of urine for estimation of free and conjugated histamine.

The volume was measured, the sample centrifuged and rapidly brought to boiling. The pH was adjusted to about 7.4. The sample was then assayed for its content of free histamine.

The hydrolysis of conjugated urinary histamine was carried out by boiling with concentrated HCl on a sand bath. 2 ml of acidified urine in a 50 ml Pyrex Erlenmeyer flask was almost evaporated by boiling on a water bath. 3 ml conc. HCl was added and the sample boiled for three hours on a sand bath as described by ROBERTS and ADAM (1950). The acid was removed by evaporating under suction over a water bath

and then 5 ml 99.5% ethanol added and evaporated three times. The dry residue was dissolved in Tyrode's solution, neutralized and assayed for histamine. The amount of conjugated histamine in the urine is represented by the difference between the total histamine after hydrolysis and the free histamine.

In control experiments we demonstrated that synthetic acetylhistamine prepared according to TABOR and MOSETTIG (1949) was completely hydrolyzed after two hours boiling on the sand bath. Boiling for one hour was inadequate, whereas boiling for two, four and six hours gave maximum yield corresponding to the stoichiometric amount of histamine. Further controls were made to examine whether boiling on the sand bath could possibly produce histamine from urinary histidine. Histidine was added to samples of urine which were then subjected to the procedures described above. In these experiments production of histamine from histidine could not be demonstrated.

Estimation and identification of histamine. The various extracts, solutions and urine samples were assayed against a standard solution of histamine acid phosphate on a strip of guinea pig's ileum suspended in a 5 ml bath of Tyrode's solution containing atropine sulphate in a concentration of 10^{-7} .

The histamine values quoted in this paper are expressed in terms of the base.

The agent measured on the gut was destroyed on incubation with a purified preparation of histaminase, prepared by Swedin's method (ARVIDSSON, PERNOW and SWEDIN 1956). Further, when mepyramine in proper doses was added to the bath for a short time, by the method of REUSE (1948), the effects of the agent studied and of histamine were equally depressed and recovered in a parallel manner. It was thus inferred that the agent assayed was histamine.

Results.

Distribution of histamine.

The distribution of histamine in three groups of rats was investigated. The results are given in Table 2. Group 1 represents eight germ free rats of both sexes, five about 120 days old and three about 300 days. Group 2 consists of six not germ free rats of both sexes, about 120 days old and fed the sterilized synthetic diet. Group 3 represents nine not germ free rats of both sexes, six about 120 days old and three about 300 days old, fed the not sterilized synthetic diet.

With the exception of the skin of the back there appears to be no difference in the distribution and content of histamine between the three groups. Because of the uncertain situation as regards the skin of the back it was decided to investigate abdominal skin,

Table 2.

Distribution of histamine in three groups of rats. Group 1: germ free, group 2: not germ free on sterilized food, group 3: not germ free on not sterilized food. The figures represent μ g histamine base per gram tissue.

No., age and sex	Ear mean of two	Skin of the back	Lung	Small intest.	Gastric mu- cosa	Gas- tro- cnem. muscle	Tongue
Group 1							
ca. 120 days old							
1 ♂.....	23.7	10.6	8.7	— ¹	—	1.7	45.8
2 ♂.....	26.4	12.3	13.8	37.1	—	3.9	49.5
3 ♂.....	25.8	11.9	7.8	12.3	30.8	4.9	27.8
4 ♂.....	22.0	13.9	6.9	9.1	16.2	4.1	23.5
5 ♀.....	19.5	11.5	5.1	10.8	56.4	4.8	30.5
ca. 300 days old							
6 ♀.....	16.3	3.6	8.0	21.0	41.4	5.6	33.3
7 ♀.....	18.6 ²	9.8	6.0	16.0	47.6	—	26.1
8 ♂.....	20.2	6.3	6.1	—	35.7	4.4	35.5
Group 2							
ca. 120 days old							
9 ♂.....	26.4	10.5	2.1	24.4	40.7	3.0	28.5
10 ♀.....	25.0	28.5	3.3	23.8	63.5	3.4	43.6
11 ♀.....	19.9	22.0	8.2	21.7	58.9	3.1	36.3
12 ♂.....	24.0	10.9	5.8	40.8	43.3	2.7	—
13 ♂.....	26.1	21.1	2.1	10.6	68.7	3.4	—
14 ♀.....	23.4	20.2	5.6	33.2	61.5	4.3	—
Group 3							
ca. 120 days old							
15 ♂.....	29.5	23.1	7.3	20.3	33.0	2.3	—
16 ♂.....	25.5	16.6	8.0	22.1	51.5	3.2	—
17 ♂.....	28.9	25.2	4.3	9.4	28.9	1.9	—
18 ♂.....	30.0	24.0	6.0	28.5	53.9	2.8	—
19 ♂.....	33.4	19.6	3.5	15.6	28.5	2.0	—
20 ♀.....	31.3	25.9	4.0	16.8	—	3.1	—
ca. 300 days old							
21 ♂.....	9.7	6.4	—	20.0	—	—	30.8
22 ♂.....	9.5	6.4	—	22.9	—	—	32.5
23 ♂.....	11.7	10.9	—	48.2	—	—	45.4

¹ — stands for sample not investigated or lost.

² Mean of two specimens of skin.

³ One ear only.

the pooled total skin, and the histamine content of the whole animal. The skin of the whole animal was weighed, minced and 0.2–0.5 g of the mince extracted for histamine. The histamine content of the soft tissues plus that of the total skin is here referred to as histamine content of the "whole animal". The values from four germ free rats of both sexes and seven not germ free

Table 3.

Histamine content in μ g base per gram tissue and total histamine in the whole animal in germ free and not germ free rats.

No., sex and age in days	Weight in g	Skin of the back	Abdominal skin	Total skin	Tongue	Soft tissue	Total histamine whole animal	Histamine per gram whole animal
Germ free								
1 ♀ 201..	200	— ¹	—	11.8	—	7.7	1 378	6.9
2 ♂ 95..	270	18.8	26.4	22.9	—	5.7	1 770	6.6
3 ♀ 166..	210	10.2	17.5	13.6	45.8	—	—	—
4 ♂ 104..	230	15.8	22.7	19.9	49.5	8.3	1 795	7.8
Not germ free								
5 ♂ 106..	235	14.3	22.2	18.2	28.5	3.4	1 163	5.0
6 ♂ 168..	370	10.0	—	11.2	43.6	—	—	—
7 ♀ 125..	220	—	—	12.8	36.3	—	—	—
8 ♀ 117..	175	—	—	12.9	—	4.8	886	5.1
9 ♂ 117..	255	—	—	15.1	—	3.9	1 219	4.8
10 ♂ 165..	360	—	19.0	17.1	—	7.9	2 635	7.3
11 ♂ 149..	350	12.7	16.4	13.6	—	5.0	1 858	5.3

¹ — stands for sample not investigated or lost.

rats of both sexes fed a not sterilized synthetic diet are presented in Table 3.

The material summarized in Tables 2 and 3 does not invite statistical treatment. However, it appears certain that the germ free rats, neither in any particular tissue examined, nor in the whole animal, contain less histamine than the not germ free rats.

Urinary excretion of histamine.

Male rats. Fig. 1 represents observations on three germ free and three not germ free rats. In the germ free rats the lowest and highest amounts of total histamine excreted in 24 hours are 9 and 23 μ g, respectively. In the not germ free group the corresponding figures are 6 and 17 μ g. These values are independent of the urine volumes. In the germ free group a large proportion of the total histamine is in the conjugated form, the ratio being subjected to considerable variations between individual rats. In the not germ free group almost all the histamine is in the conjugated form.

Female rats. So far, only one germ free female could be investigated closely as shown in fig. 2. Numerous not germ free females

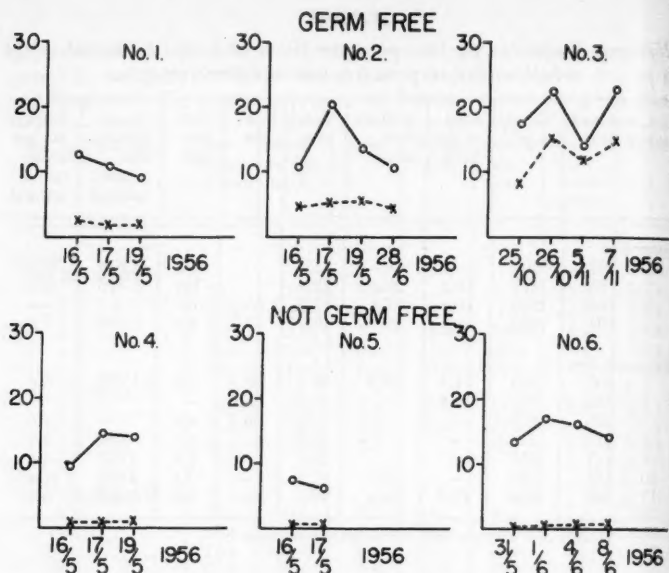


Fig. 1. Urinary excretion of histamine in $\mu\text{g}/24$ hr in three germ free and three not germ free male rats, all fed a histamine free sterile diet. x-x-x- free histamine, $\circ-\circ-\circ$ total histamine.

have been examined of which two typical ones are included in fig. 2. The germ free female excreted large amounts of both free and conjugated histamine. In not germ free females the daily output of histamine is at about the same rate as in males. There is, however, the striking difference that the not germ free females excrete histamine almost exclusively in the free form.

Nature of the conjugated histamine. A total of 65 ml urine was collected from a germ free female rat. The first step of purification from the urine depended on the extraction of histamine by butanol at an alkaline pH as described by MCINTIRE, ROTH and SHAW (1947) and the subsequent reextraction into the aqueous at an acid pH. The final extract to be applied on the paper strip was prepared according to TABOR and MOSETTIG (1949). Paper chromatography was carried out in the system butanol- H_2O -glacial acetic acid. The spots were developed with diazotized p-bromoaniline as described by URBACH (1949). On running over night on strips of No. 1 WHATMAN paper acetylhistamine and the

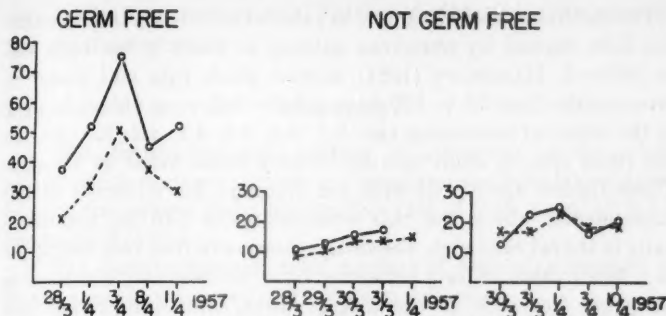


Fig. 2. Urinary excretion of histamine in $\mu\text{g}/24$ hr in one germ free and two not germ free female rats. x---x---x free histamine, o---o---o total histamine.

urinary conjugate advanced at the same rate. No attempt was made to estimate quantitatively by chromatography the amount of urinary acetyl histamine.

Absence of histamine in faeces. The faeces of the various groups of rats did not contain free or conjugated histamine in measurable quantities.

Discussion.

To our knowledge, except for a brief note (GUSTAFSSON and KAHLSON 1956), this is the first recorded study on distribution and metabolism of histamine in germ free reared mammalia. Since these rats from weaning onwards were fed a histamine free sterilized diet it is obvious that every molecule of histamine present in their tissues is endogenous in origin, exogenous sources being safely excluded.

In not germ free rats fed the same diet, which by its casein content provides histidine for bacterial decarboxylation, the distribution and total content of histamine appears to be the same as in the germ free animals. This uniformity between the two groups is consistent with the notion of a regulatory mechanism controlling the histamine content of tissues as suggested by HAEGER, KAHLSON and WESTLING (1953) and in accord with SCHAYER's (1952) observation that exogenous C^{14} -histamine is not retained in the tissues for even four hours.

The distribution of histamine in rats fed a diet rich in histamine has been studied by numerous authors to which a few only will be referred. HARDWICK (1954) minced whole rats and found in seven adults from 70 to 320 days old the following values in $\mu\text{g/g}$ in the order of increasing age: 6.1, 5.7, 9.4, 4.1, 4.2, 5.7, 4.1. In the flank skin of adult rats he found a mean value of 15 $\mu\text{g/g}$. These figures agree well with our findings. In WILSON's (1954) experiments, where the rats received about 340 μg histamine daily in the rat cake diet, the small intestine in four rats contained as a mean value 14 $\mu\text{g/g}$ histamine base, considerably less than in our germ free rats. In the tongue MOTA, BERALDO, FERRI and JUNQUEIRA (1956) found 14.5 and WEST (1956) 36 $\mu\text{g/g}$, which latter figure agrees with ours.

The urinary excretion of histamine as observed in this study presents surprising features. First, in male rats, the amount of total histamine excreted daily is about the same in germ free and not germ free rats. This indicates that either the amount of histamine derived from the intestines by bacterial decarboxylation is very small or that such histamine, after absorption, is not excreted as free or conjugated histamine in quantities sufficient to produce a distinct difference in the amount of total histamine between the two groups. Further, the germ free rat does conjugate histamine, specifically acetylate, as demonstrated by paper chromatography. The not germ free male conjugates to the extent that almost all urinary histamine is in the conjugated form.

In the female, most surprisingly, the germ free conjugates whereas the not germ free rat does not or very poorly so. The differences between the various groups in the excretion of conjugated histamine can not be explained without further experiments.

The urinary excretion of histamine is now liberally used in studies on the metabolism and release of histamine. A diet rich in histamine will obscure the picture for the following reasons. First, the metabolism of exogenous and endogenous histamine may possibly follow different metabolic paths. Second, large amounts of urinary histamine derived from the food are likely to swamp small increases in urinary histamine derived from increased release of endogenous histamine. Studies on the normal turnover and physiological liberation of histamine are likely to be more yielding in rats fed a histamine free diet. Such animals, particularly the males, are in the urinary excretion of histamine

very similar to germ free rats. Further studies under this assumption are in progress.

Summary.

1. The distribution and content of histamine in the whole animal and the urinary excretion of histamine has been investigated in germ free reared and not germ free rats of both sexes, all fed a histamine free diet.

2. The distribution and total content of histamine appears to be the same in germ free and not germ free rats.

3. In male rats the daily excretion of total histamine is about equal in germ free and not germ free rats, 9—23 $\mu\text{g}/24$ hr in the former group, 6—17 in the latter. In germ free male rats a large proportion of the total histamine is in the conjugated form in the not germ free almost all the histamine is conjugated.

4. Female germ free rats excrete both free and conjugated histamine, whereas not germ free females excrete histamine almost exclusively in the free form.

5. Evidence is presented that the conjugate in the urine of a germ free female may be acetylhistamine.

6. The consequences of the present observations for further studies of the metabolism and release of endogenous histamine is discussed.

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On the Contraction of Glycerol-extracted Muscle Fibre Bundles under Highly Isometric Conditions.

By

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The glycerol-extracted muscle fibre probably represents the contractile mechanism of the muscle in simplified form. The course of contraction of such a muscle model is still a complicated process, among other reasons because the contraction-inducing substance, adenosinetriphosphate (ATP), must be supplied to the fibres from the outside. The Weber school (BRIGGS and PORTZEHL 1957, HASSELBACH 1956, HEINZ and HOLTON 1952, PORTZEHL 1952, G. ULBRECHT and M. ULBRECHT 1953, 1954, A. WEBER 1951, A. WEBER and H. H. WEBER 1951) has attempted to simplify the situation by working with single muscle fibres, but, as will be shown later, this has failed to solve the problem. The use of glycerol-extracted myofibrils (PERRY 1954, PORTZEHL 1954, 1957) is a further attempt to eliminate the diffusion problem, but unfortunately no isometric studies are possible with myofibril material. In a number of investigations bundles of glycerol-extracted muscle fibres have been used as test objects instead of single fibres (BENDALL 1953, 1954, BOWEN 1952, BOWEN and KERWIN 1955, BOZLER 1951, 1952 a, 1952 b, 1953, 1954 a, 1954 b, 1956, BOZLER and PRINCE 1953, EDMAN 1953, 1956, GOODALL 1956, GOODALL and A. G. SZENT-GYÖRGYI 1953, HASSELBACH 1956, KOREY 1950, LAKI and BOWEN 1955, LORAND and MOOS 1956, MATOLTSY and VARGA 1950, MOOS and LORAND 1957, MUGIKURA et al. 1956, NEWBOLD and ROSE 1957, RANNEY 1954 a, 1954 b, 1955 a, 1955 b,

SZENT-GYÖRGYI 1949, VARGA 1950). Fibre bundles have the advantage of being easier to handle than single fibres. No detailed analysis of the course of the contraction in glycerol-extracted muscle fibre bundles has yet been published, however. Several of the earlier studies in this field have treated isotonic contraction. In isotonic contraction an inactive, ATP-free nucleus of the fibre must inhibit the shortening process of the active parts of the fibre. The same interference source must also be important in "isometric" recording with the majority of the methods used earlier as a more or less pronounced shortening of the fibres has been unavoidable. Only recording with a high degree of isometry can eliminate interference from inactive parts of the fibre during the contraction. Such methods for isometric recording have been described by RANNEY (1954 c) and DETTLI and BING (1956).

The present study presents a more detailed analysis of the factors that might conceivably be involved in the isometric contraction of glycerol-extracted muscle fibre bundles. The experimental portion of the work is based on a new method that makes it possible to record the contraction under highly isometric conditions. The results described in a preliminary report (EDMAN 1956) were obtained with the apparatus presented here.

Methods.

The glycerol extraction of the muscle fibres was done according to the method described by SZENT-GYÖRGYI (1949). The details of the extraction procedure have been published earlier (EDMAN 1956). The extracting solution consisted of 50 % aqueous glycerol with 10^{-2} M sodium phosphate buffer (pH 7.0). Only fibres extracted 8 to 21 days were used in these studies. After the glycerol extraction a KCl solution containing 10^{-2} M veronal buffer with pH 7.3 and total K^{+} concentration 10^{-1} M was used consistently. The buffer also contained 10^{-3} M $MgCl_2$. This buffer solution was used as solvent for all substances studied.

After the extraction the muscle fibre bundles were dissected at room temperature to successively finer bundles, first in the glycerol solution described above and finally in the veronal buffer. This and all further manipulation before the start of the experiment was carried out under fluid immersion in a Petri dish. The prepared fibre bundle was examined microscopically before use with a long distance working attachment (Newton & Co. Ltd.) at 200 times magnification. Fibre bundles exhibiting any signs of tissue damage or broken continuity were discarded. The dimensions of the fibre bundles were determined by means of an ocular micrometer calibrated against an objective micrometer. The

cross section of a fibre bundle presents an irregular surface, most nearly resembling an elliptic or rectangular form. For this reason the maximum and the minimum diameters were measured for each fibre bundle, and in order to obtain these two measurements most accurately the fibre bundle was twisted one turn along its longitudinal axis before measurement. (For data on the dimensions of the fibre bundles used, see Results.) Hereafter, when thickness is given it refers to the smallest diameter of the fibre bundle.

For the isometric recording it is necessary that the actual mounting arrangements for the fibre bundle should not introduce any compliance that could interfere with the isometry. For this reason the fibre bundles were mounted between two platinum loops in the manner shown in the detail sketch in figure 1. The weight of the upper loop is 25.0 mg (wire diameter 0.20 mm) and of the lower loop 7.5 mg (wire diameter 0.15 mm). The fixation of the muscle fibre bundle between these two loops was done in the buffer solution in a Petri dish in conjunction with the final dissection of the fibres. An 0.08 mm thick nylon thread was used for tying the bundle to the platinum wire. At the site of the knots the fibre bundle becomes compressed, but effective attachment is achieved without any microscopically observable break in the continuity. The length of the fibres between the knots was 10 mm. Due to the remarkable parallelism of the fibres in the psoas muscle it is relatively easy to prepare a 10 mm long fibre bundle with constant measurements for maximum and minimum diameter throughout its entire length.

The pH determinations were performed with a glass electrode. All solutions were prepared with water that had been twice distilled in Pyrex glass. The glass vessels were also washed in twice distilled water.

The chemicals used were of the following qualities: NaH_2PO_4 , Crystal, Baker's Analyzed; Na_2HPO_4 (Sörensen), E. Merck; Veronal, Diethylbarbituric acid, Crystal, Ph.S.XI; NaOH, pro analysi, Elektrokemiska A.B. Bohus; KOH, Titrisol, E. Merck; HCl, Titrisol, E. Merck; K_2SO_4 , Crystal, meets A.C.S. specifications, Baker's Analyzed and pro analysi, E. Merck; Glycerol, Glycerinum bidestillatum 1.23, pro analysi, E. Merck; MgCl_2 , meets A.C.S. specifications, Baker's Analyzed.

ATP was obtained from Schwartz Laboratories in the form of di-barium salt, "chromatographically pure", Lot. No. 5204. The purity of the ATP preparation and the concentration of ATP, ADP and AMP was examined with ion exchange chromatography on the formate form of Dowex 1 resin ($\times 10$, 200—400 mesh) according to the method described by HERBERT, POTTER and TAKAGI (1955). The column volume was $0.882 \text{ cm}^2 \times 17.0 \text{ cm}$. For elution of AMP and ADP 2.5 N formic acid was used and after that ATP was eluted by 1.25 N ammonium formate. To make it more easy for possible impurities to separate from the ATP fraction one examination was performed, where the formic acid was followed by about 6 columnar volumes of 0.7 N ammonium formate succeeded by 1.25 N ammonium formate. According to HERBERT, POTTER and TAKAGI (1955) 0.7 N ammonium formate will elute nucleotides such as uridine phosphates, but not ATP. Of the ATP substance 51 mg was dissolved in 6 ml double distilled water, acidified

with HCl to about pH 2. A small fraction of the substance was insoluble and was removed by centrifugation. Of the solution 1 ml was put on the ion exchange column, and the rest of the solution was used as standard (= 100% nucleotide concentration) in the spectrophotometric determinations and for estimation of the total phosphorus content. The optical density of the nucleotide solutions was measured in a Beckman model DU spectrophotometer with quartz cuvettes (1 cm light path). The readings were performed at 260 $m\mu$ in the case of the standard solution and the ATP fraction (pH \sim 7–8) and at 257 $m\mu$ in the case of ADP and AMP (pH \sim 2). The values used for the molar absorption index were 15.4×10^3 for ATP and the standard solution and 15.0×10^3 for ADP. The absolute concentration of adenosine nucleotides in the original solution was obtained spectrophotometrically with the molar absorption index as point of departure and also by determination of the total phosphorus content according to K. C. SCHEEL (1936) after wet combustion with a mixture of H_2SO_4 - HNO_3 - $HClO_4$. The values of the absolute and relative nucleotide concentrations are the means of two different examinations. According to the spectrophotometric determination the absolute nucleotide concentration of the original solution was 0.90×10^{-2} M and, according to the phosphate analysis, 0.92×10^{-2} M, on the assumption that all phosphorus was referable to ATP. Of the total nucleotide concentration 89% was ATP and 4% ADP with only traces of AMP. An unknown fraction was eluted by the 0.7 N ammonium formate or was found immediately before the ATP fraction when the 1.25 N ammonium formate was used directly after the 2.5 N formic acid. The optical density of the unknown fraction (15.5 ml total volume, pH \sim 2) was 0.065 at 257 $m\mu$. This will amount at most to about 1% of the total nucleotide content, assuming the molar absorption index of the unknown substance to be 10×10^3 . For calculation of the ATP concentration in the rest of this paper, the concentration of ATP in the aforementioned original solution was considered to be $0.89 \times 0.91 \times 10^{-2}$ M.

K_4 ATP was freshly prepared daily from Ba_2 ATP in the manner described in an earlier work (EDMAN 1953). In the present study the solution of K_4 ATP neutralized to pH 7.3 (as measured with Merck's indicator paper) and in a quantity of 8–10 ml was buffered with the aforementioned veronal buffer to 60 ml total volume. This solution comprised the stock solution for one day's experiments and contained 1.5×10^{-3} M ATP. It was stored at room temperature, and no change in its activity during storage was observed in the contraction experiments.

The experiments were performed at room temperature (20–22°C).

Description of the apparatus for isometric recording.

See figure 1. The apparatus works on the principle of the torsion balance. A horizontally suspended steel wire is rigidly attached at one end (A), but able to be rotated through the other point of attachment (B). The wire can also be rotated via a lever (C) at the middle of the

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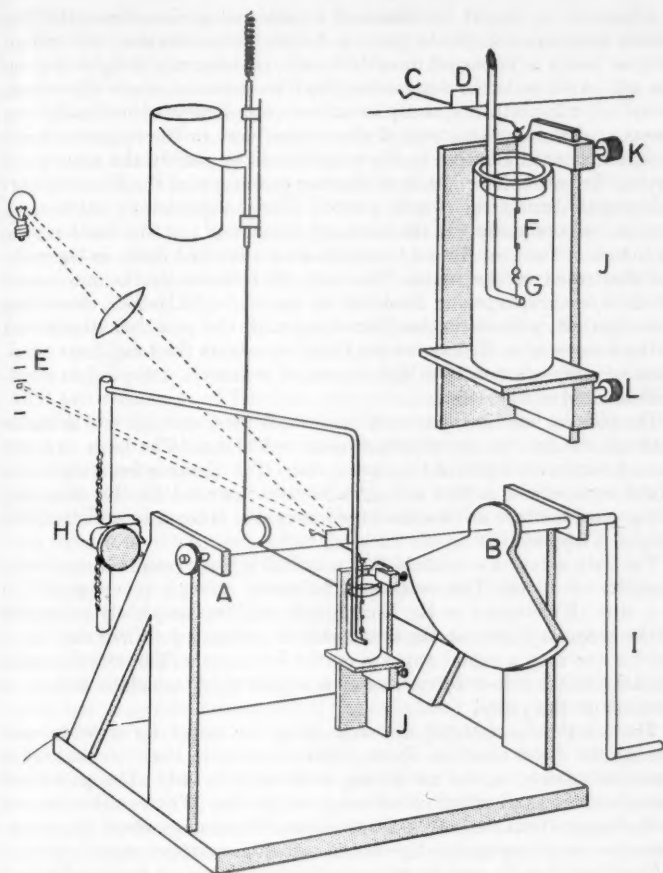


Fig. 1. Apparatus for isometric recording.

A-B: Torsion wire. C: Lever. D: Mirror. E: Calibrated scale.

F: Muscle fibre bundle mounted between two platinum loops.

G: Platinum hook for the lower fixation of the fibre bundle.

H: Rack and pinion drive.

I: Arm for the compensatory rotation of the torsion wire.

J: Metal guide.

K: Stop clamp.

L: Support for fixation of the bath vessel.

torsion wire. A mirror (D), mounted on the lever projects a light spot onto a calibrated scale (E). By means of this device rotation of the torsion wire can be observed. The muscle fibre (F) is applied vertically between the lever and a platinum hook (G) fused into a glass rod, which

is adjustable in height by means of a rack and pinion drive (H). The muscle fibre can thereby be given a definite initial tension, the magnitude of which is indicated directly by the position of the light spot on the calibrated scale. In contraction the fibre seeks to rotate the torsion wire, but this rotatory component is continuously compensated by rotation of the movable end of the torsion wire in the opposite direction so that the light spot on the scale is held in exactly the same place during the entire determination. Hereby the length of the fibre remains unchanged during the whole period. The compensatory rotation is carried out manually via the rotatory attachment of the torsion wire by means of the arm (I) and is recorded on a smoked drum in the manner illustrated in the figure. The cord that transmits the movement to the kymograph pen is attached at one end of a groove describing a circular arc with its centre coinciding with the point of attachment of the torsion wire. The curve obtained represents the tension development of a contraction at a high degree of isometry. A typical example is illustrated in figure 2.

The torsion wire is a 0.12 mm thick steel wire, and the free distance between the two points of attachment is 130 mm. The lever arm (C) has a hook on each side of the torsion wire. The distance from the hooks to the torsion wire is 23.0 mm. One hook is intended for the mounting of the muscle fibres as described earlier, on the other a suitable counter weight is applied.

The bath solution is contained in a cylindric glass vessel always in the quantity of 40 ml. The vessel can be raised along a metal guide (J) to a stop (K) situated so the fibre bundle will be completely immersed in the bath, as shown in the detail sketch in figure 1. When the vessel reaches the stop a signal appears on the kymogram. The vessel can be fixed in this position by means of a support (L), which is locked in position on the guide.

The solution is changed by exchanging the vessel for another containing the fresh solution. During this manoeuvre the fibre bundle is suspended freely in the air during 4—5 seconds only. The procedure permits a rapid and effective exchange of solution. The vessel is moved in the longitudinal axis of the fibre bundle. No damage could be established as occurring under the experimental conditions described.

The fibre bundle was given an initial tension of 17.2 mg, of which 7.2 mg was exerted by the lower platinum loop. The rest of the initial tension was obtained by lowering the lower attachment of the fibre bundle by means of the rack and pinion drive. The magnitude of the applied tension can be read directly on the calibrated scale.

In calibration of the scale a fibre bundle with its platinum loops was suspended freely in the bath solution from the lever and balanced with a suitable counter weight. The position of the light spot after extra loading of the lever with known weights was subsequently marked on the scale.

The maximum compensatory rotation of the torsion wire is 90° , which makes it possible to record a maximum of about 150 mg tension development by a fibre bundle. Within this operating range of the torsion wire the compensatory rotation is directly proportional to the tension recorded.

The procedure in an experiment is as follows:

The fibre bundle, which has been continually immersed in solution in a Petri dish, is suspended from the lever (fig. 1). The vessel with the bath solution is immediately raised along the metal guide to the stop and fixed in that position by means of the support, which is locked on the metal guide. Thus, the fibre bundle is not permitted to hang exposed to air for more than 10 seconds at most. A suitable counter weight is applied to the lever on the opposite side of the torsion wire. The lower loop of the fibre bundle is then hooked onto the platinum hook, and an initial tension is applied to the fibre bundle according to the foregoing description. The contraction is started by exchanging the first glass vessel with bath solution for another containing the same solution, but with ATP. This exchange of the bath solution is done with the left hand and can be carried out without distracting the attention from the light spot on the scale. The development of tension does not begin until after a certain latent period following the immersion in ATP (see section 1. of Results), and is indicated by an upward movement of the light spot on the scale. The compensatory rotation of the torsion wire, as already described, is performed with the right hand through moving the arm (I) in a manner such that the light spot on the scale is held at the same level during the course of the experiment. With this arrangement it is possible to record the course of a contraction from 0.1—0.2 seconds after the start signal with the ATP concentrations used here, i. e. up to 1.1×10^{-3} M.

The isometry in this recording arrangement might conceivably be disturbed by the following factors:

1. Inexact compensation of the developed tension by means of the manual servo device.

In order that a change in the contraction status of the fibres may be perceived, a certain movement of the indicating light spot on the scale must first be permitted to occur. Accordingly, the light spot must be permitted to move within a certain range on each side of the initial level during the course of a contraction. This means that the fibres are somewhat stretched during certain brief intervals and somewhat shortened in others in relation to their initial length. The deviation from isometry thus resulting amounts, however, to not more than 0.01 mm.

2. Flexing of the torsion wire due to the traction of the fibre bundle.

3. Faulty centering of the torsion wire, which causes a vertical displacement of the wire when it is rotated.

The sources of error given in points 2 and 3 were investigated by means of an ocular micrometer at 30 times magnification. When the torsion wire's lever carries a load of 150 mg, which approximately corresponds to the maximum force that can be recorded, the lowering of the torsion wire at the site of the lever is less than 0.005 mm. Faulty centering of the torsion wire caused the middle part of the wire to be lowered in the recording of a contraction. This lowering did not exceed 0.05 mm at the site of the lever.

The total deviation from isometry thus amounts to not more than 0.06 mm, which means that the fibre bundles used were permitted to

shorten by not more than 0.6 % of their length during the course of the experiment.

The torsion rigidity of the wire was checked after about every fourth experiment. In this check the rotation of the wire required to compensate for a 10 mg weight placed on the lever was recorded on the kymogram. Constant values were obtained during several months.

The apparatus can also be used for strictly isotonic recording. In this case the torsion wire is held unchanged during the course of the contraction by displacing the lower point of attachment of the fibre bundle (G) upwards by means of the rack and pinion drive (H) so that the indicating light spot on the scale is held still. The movement of the drive is recorded on the kymograph. The same precision can be attained as in the isometric determinations, but since the isotonic contraction is considerably slower than the isometric, this means of recording isotonic contraction is inconvenient.

The elasticity of the fibres can also be measured with the apparatus described. The fibre bundle is stretched to the extent desired by rotation of the torsion wire by means of the arm (I). The magnitude of the stretching of the fibre bundle can be read from the position of the light spot on the scale. The opposing rotation of the torsion wire is a measure of the increase in tension in the fibre bundle induced by the stretching and is recorded in the aforementioned manner on the kymograph.

Results.

The same experiments were the basis for the results under 1, 4 and 5 in the following, where the ATP concentration was varied over a relatively wide range ($0.8-11 \times 10^{-4}$ M). The minimum transverse dimension of the fibre bundles was $75-140 \mu$, and the maximum transverse dimension $145-250 \mu$. The fibre bundles used originated from one single muscle preparation with the exception of the experiments with ATP concentrations 7.5 and 9.0×10^{-4} M, where in addition fibre bundles from one other muscle preparation were used. The results under 2 and 3 represent a series of experiments in which the ATP concentration was held constant at 2.3×10^{-4} M, but in which the transverse dimensions of the fibre bundles were varied (see following). The fibre bundles in these experiments originated from one single muscle preparation.

In figure 2 a typical course of an ATP-induced isometric contraction of a glycerol-extracted muscle fibre bundle is illustrated. The tension development starts with a certain latent period after the ATP administration and grows relatively rapidly to about 50 % of the final magnitude of the contraction. In the subsequent



Fig. 2. A typical course of isometric contraction of a glycerol-extracted psoas fibre bundle induced by 3.8×10^{-4} M ATP. The short base line before the contraction represents the initial tension (15 mg) of the bundle. At the arrow the fibre bundle is immersed in the ATP solution. The tension development (downwards) begins after a short latency. Time marks: seconds. Breaks in time marks represent minutes. Ordinate: Tension development in mg. Cross section of the fibre bundle: $89 \times 173 \mu$. The tracing reduced 6 times.

course the contraction curve flattens out successively and approaches the final level asymptotically.

1. Latency.

The latent period, *i. e.* the interval between the administration of ATP and the start of the contraction, decreases with an increase in the ATP concentration. This behaviour is clearly shown in figure 3 for latent periods exceeding 0.1–0.2 seconds. Shorter periods than these cannot be recorded with the method used.

2. The diffusion of ATP into the fibre bundle.

A comparison of the contraction course for fibre bundles of varying thickness at the same ATP concentration shows the maximum thickness that can be used if even the centre is to be reached by ATP in amounts sufficient to induce tension. Figure 4 presents an example of such a procedure. This figure shows several typical contraction curves with the same ATP concentration, 2.3×10^{-4} M, but with varying thickness of the fibre bundles (for further details see figure text).

For the thinnest fibre bundles, with the smallest dimension about 45μ , the contraction ended after only 10–20 seconds, as is evident from figure 4. The thicker the fibre bundle, the later constant tension is attained, and for 80 – 90μ bundles the contraction curve reached a constant level only after about 100 seconds. For thicker fibre bundles the tension is not generally completely steady even 100 seconds after the start, but the additional tension developed later is insignificant. The fact that the tension for a thin fibre bundle is stable at a time when the contraction has not yet reached completion for a thicker fibre bundle must mean that ATP has reached the centre of the thin fibre

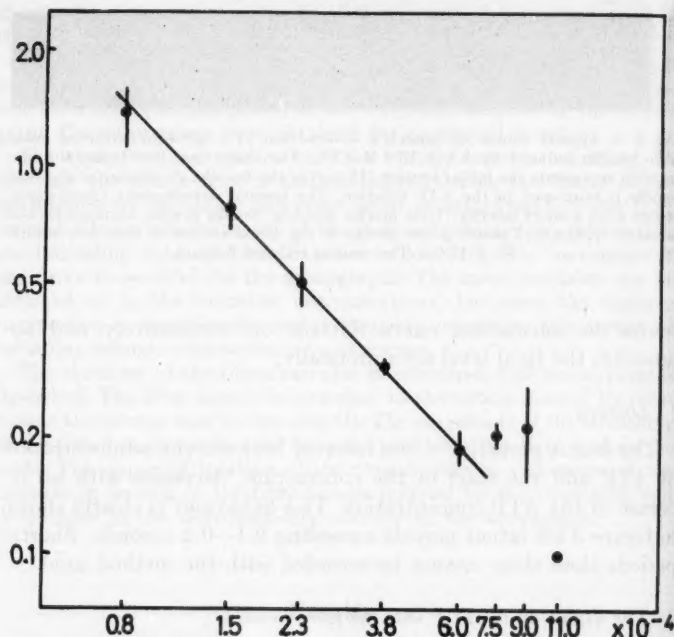


Fig. 3. Latency between the moment of immersion of the fibre bundle into ATP and the beginning of the tension development at different concentrations of ATP. Ordinate: Latency in seconds. Abcissa: Molar concentration of ATP. Each symbol represents the mean of 6—13 experiments, except the lowest point, which represents 4 identical readings. The standard error of the mean values is indicated with a bar. Cross section of the fibre bundles: $75\text{--}140 \times 145\text{--}250 \mu$.

bundle. The results show that ATP with 2.3×10^{-4} M external concentration can penetrate to the centre of $80\text{--}90 \mu$ thick fibre bundles. This will be discussed in detail later.

3. *The relation between final isometric tension and the thickness of the fibre bundles.*

Figure 5 illustrates the close relation between the final isometric tension and the circumference of the fibre bundle. The experiments were carried out with constant ATP concentration (2.3×10^{-4} M), but with variation of the thickness of the fibre bundles. In all experiments the tension 5 minutes after the start of the contraction was chosen as the final tension. This is to a certain degree an approximation inasmuch as the increase in tension is

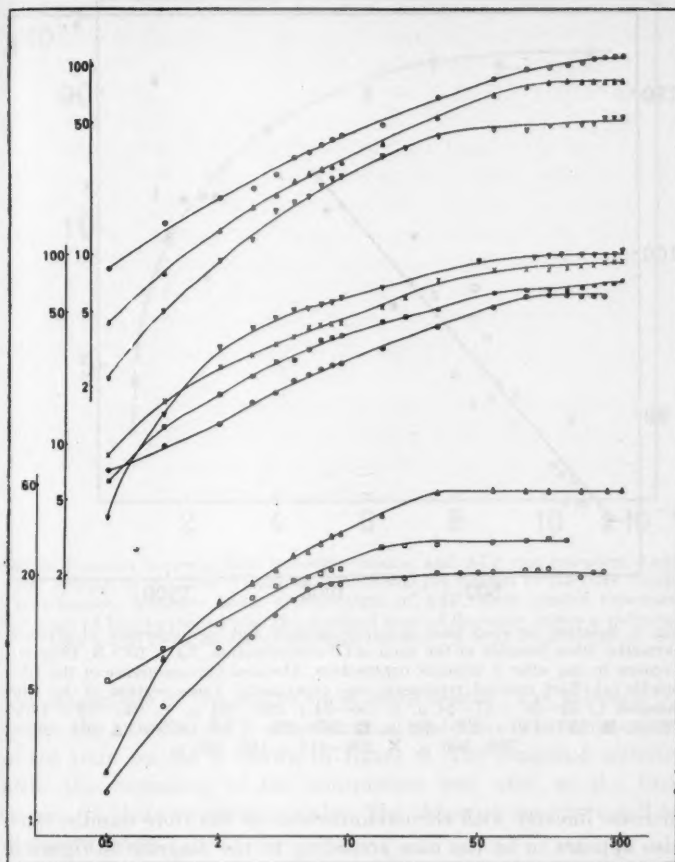


Fig. 4. Comparison between isometric contractions of glycerol-extracted fibre bundles of different thickness at the same ATP concentration, 2.3×10^{-4} M. Ordinates: Tension development in mg. Abcissa: Time in seconds after immersion into ATP. Each curve represents the contraction of one fibre bundle. Cross section of the fibre bundles: \odot 187 \times 280 μ , \blacktriangle 126 \times 262 μ , ∇ 84 \times 150 μ , \blacktriangledown 150 \times 206 μ , \times 150 \times 215 μ , \blacksquare 94 \times 145 μ , \bullet 94 \times 126 μ , \triangle 56 \times 140 μ , \square 47 \times 56 μ , \circ 42 \times 47 μ .

not entirely finished even after 5 minutes for the very thickest bundles.

Because of the limited depth of penetration of ATP into a fibre bundle it might be expected that the final tension would

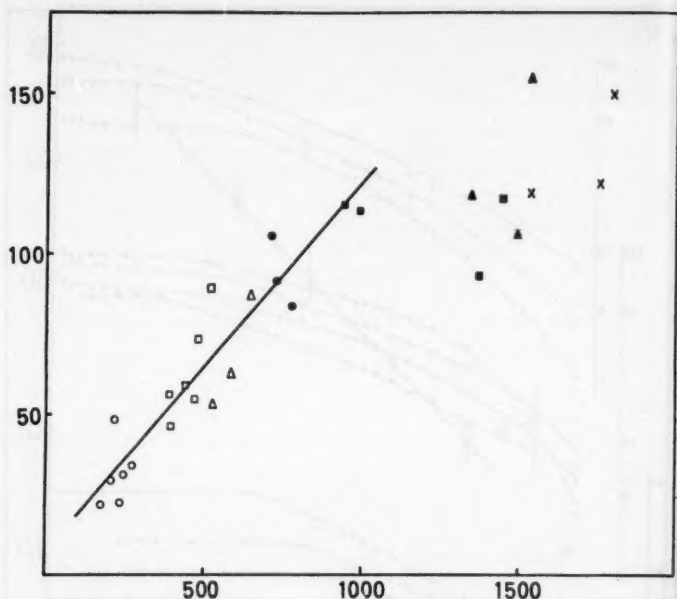


Fig. 5. Relation between final isometric tension and circumference of glycerol-extracted fibre bundles at the same ATP concentration, 2.3×10^{-4} M. Ordinate: Tension in mg after 5 minutes' contraction. Abscissa: Circumference of the fibre bundle (μ). Each symbol represents one experiment. Cross section of the fibre bundles: \circ 42–51 \times 47–84 μ , \square 56–94 \times 126–164 μ , \triangle 94–112 \times 150–229 μ , \bullet 126–150 \times 206–262 μ , \blacksquare 187–252 \times 281–477 μ , \blacktriangle 262–281 \times 393–505 μ , \times 290–411 \times 449–505 μ .

increase linearly with the circumference of the fibre bundle. Such also appears to be the case according to the diagram in figure 5. The values obtained can be fitted by a straight line with the exception of the group of results representing extremely thick fibre bundles. Further discussion of the results is given later.

4. The relation between final isometric tension and ATP concentration.

The tension developed by each individual contractile element most probably depends on its contractility and on the ATP concentration. In the case of a whole fibre or a fibre bundle the ATP concentration, however, is governed by diffusion phenomena that determine the concentration distribution of ATP in the fibre and

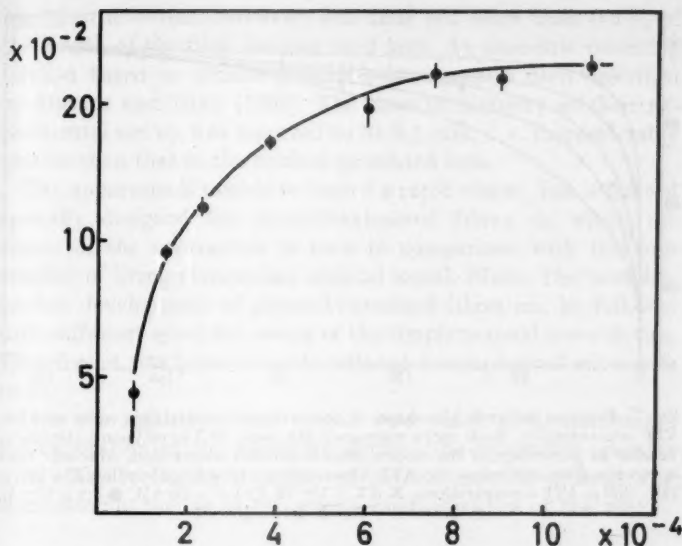


Fig. 6. Relation between final isometric tension and ATP concentration. Ordinate: Tension in mg after 5 minutes' contraction per micron of the fibre bundle circumference. Abscissa: Molar concentration of ATP. Each symbol represents the mean of 4-14 experiments. The standard error of the mean values is indicated with a bar. Cross section of the fibre bundles: $75-140 \times 145-250 \mu$.

the magnitude of the active cross section area. The relation between the external ATP concentration and the maximum tension of the fibre bundle is shown in figure 6. The tension 5 minutes after the beginning of the contraction was used as the final tension in these experiments also. The shape of the curve will be discussed later.

5. *The relation between the shape of the isometric contraction curve and the ATP concentration.*

The shape of the isometric contraction curve varies with the ATP concentration. This is illustrated in figure 7, where the tension development during the course of the contraction is given as percentage of the final tension. The tension 5 minutes after the start of the contraction was used, as in the foregoing, as the final tension. It is evident that the contraction curve has a steeper initial slope at higher ATP concentrations. For instance, 80 % of the final tension is attained approximately 15 times more

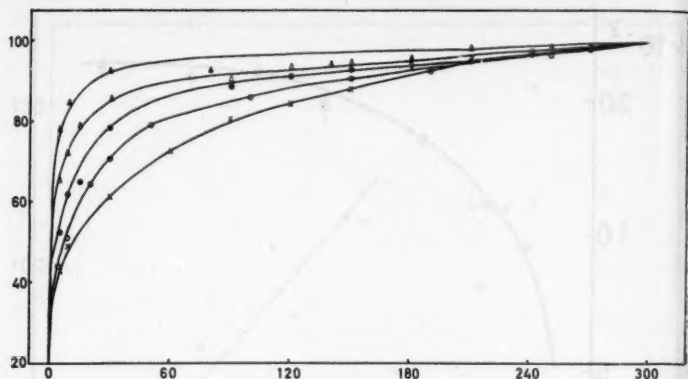


Fig. 7. Relation between the shape of the isometric contraction curve and the ATP concentration. Each curve represents the mean of 3 experiments. Ordinate: Tension as percentage of the tension after 5 minutes' contraction. Abscissa: Time in seconds after immersion into ATP. Cross section of the fibre bundles: $75-140 \times 145-250 \mu$. ATP concentrations: $\times 0.8 \times 10^{-4} \text{ M}$, $\circ 1.5 \times 10^{-4} \text{ M}$, $\bullet 3.8 \times 10^{-4} \text{ M}$, $\Delta 9.0 \times 10^{-4} \text{ M}$, $\blacktriangle 1.1 \times 10^{-3} \text{ M}$.

rapidly with $1.1 \times 10^{-3} \text{ M}$ ATP than with $0.8 \times 10^{-4} \text{ M}$ ATP concentration. The slope of the contraction curve is an expression of the rate of contraction. With the contraction curves illustrated in the manner shown in figure 7 the slope is independent of the absolute magnitude of the tension development during the course of the contraction and is instead an expression of the portion of the final tension developed per time unit. Further discussion of the rate of contraction and the shape of the contraction curve will be presented below.

Discussion.

The method described differs from earlier published methods for isometric recording. The tension development in the fibres is compensated continuously by means of the servo device described earlier. According to conventional isometric technique, on the other hand, the fibres are allowed to contract against an elastic resistance, and it is this more or less negligible shortening that is recorded and comprises a measure of the "isometric" tension development. As stated earlier under Methods, small deviations from pure isometry are unavoidable even with the method described herein. The error in isometry is a matter of

less than 0.06 mm, however, and thus not more than 0.6% of the length of the fibre bundles used here. An isometric recording method based on similar principles has recently been described by DETTLI and BING (1956). The error in isometry in their experimental set up was reported to be 0.1 mm, *i. e.* inconsiderably greater than that in the method presented here.

The apparatus is unable to record a rapid course, but is instead specially designed for glycerol-extracted fibres, in which the course of the contraction is slow in comparison with the contraction of living mammalian skeletal muscle fibres. The isometric tension development of glycerol-extracted fibres can be followed with sufficient speed by means of the simple manual servo device. Therefore it was possible to do without a complicated automatic servo.

It is the rigidity of the torsion wire which alone determines the calibration of the recording device. The apparatus operates with stability during a period of several months without any demonstrable change in the torsion characteristics of the wire.

General comments on the ATP-induced contraction of glycerol-extracted muscle fibres.

Several factors are important in the contraction of glycerol-extracted muscle fibres. The contraction-inducing substance, ATP, must be administered from the outside and must diffuse in to the contractile element. As demonstrated by HASSELBACH (1952), ATP has only a limited depth of diffusion into the glycerol-extracted muscle fibre (see later discussion). This must mean that ATP is continuously the object of enzymatic breakdown through the ATPase in the muscle fibre so that a steady state occurs between breakdown and diffusion of ATP into the fibre. In this respect the ATPase activity thus inhibits the contraction of the glycerol-extracted muscle fibres.

In the opinion of many, however, splitting of ATP is necessary for the occurrence of a contraction of a glycerol-extracted muscle fibre. This conception is supported by experiments of BENDALL (1953). According to this author a variation of the Mg^{++} concentration causes changes in the rate of shortening of glycerol-extracted muscle fibres (during the first two minutes of an isotonic contraction) closely correlated with changes in the ATPase activity of fibre material (during 4 minutes' incubation with ATP). BOZLER

and PRINCE (1953) have shown, that the spontaneous relaxation observed in their experiments after the ATP-induced contraction, was accompanied by a decrease of the ATPase activity. As demonstrated by PERRY (1954, 1956) a small and in itself insufficient quantity of ATP can induce shortening of glycerol-extracted myofibrils if continuous splitting of ATP is made possible by the presence of a creatinephosphate-creatinephosphokinase system, which rephosphorylates the ADP formed. Similar results were attained by BOZLER (1953) regarding the tension development of glycerol-extracted muscle fibre bundles. MOOS and LORAND (1957) have recently shown, that ATP in low concentrations (10^{-5} M) is able to induce isometric contraction of glycerol-extracted psoas fibres in the presence of a phosphoenolpyruvate-pyruvatephosphokinase system. This enzyme system also has the ability to rephosphorylate ADP. These observations by PERRY, BOZLER and MOOS and LORAND seem to comprise the best argument thus far for the contention that splitting of ATP is of fundamental significance for the occurrence of contraction due to ATP. Further support for this opinion is given in the reviews of H. H. WEBER and PORTZEHL (1952, 1954). There are reports, however, showing a discrepancy between the effect of a substance on the rate of splitting of ATP on the one hand and on the rate of shortening of actomyosin threads (BOWEN 1952: Ca^{++} , Co^{++}), glycerol-extracted muscle fibre bundles (BOWEN and KERWIN 1955: K^{+}) or myofibrils (PORTZEHL 1954: Mersalyl) on the other. TURBA and KUSCHINSKY (1952) found a discrepancy between the effect of oxarsan and fuadin on the splitting of ATP and the magnitude of the actomyosin syneresis. Contradictory to these results MUGIKURA et al. (1956) found the inhibition of the actomyosin syneresis by oxarsan to be closely correlated with a decrease of the ATPase activity.

According to theories presented by MORALES et al. (1955) ATP exerts its contraction-inducing effect through being bound to the contractile element, the splitting of ATP being of secondary importance. It is not inconceivable that certain characteristics in the contractile element influencing its relation to ATP, *e. g.* the ATPase activity and the ability to bind ATP or its breakdown products, vary according to the type of the contraction: isometric or isotonic, with or without load. It is further possible that the hydrolysis of ATP or the binding of ATP to the contractile element vary with the degree of shortening or tension, as

earlier pointed out by G. ULBRECHT and M. ULBRECHT (1953) and MORALES et al. (1955). These questions are important in a more detailed analysis of the course of the contraction of glycerol-extracted muscle fibres, but unfortunately cannot as yet be answered.

The course of the isometric contraction of a glycerol-extracted muscle fibre bundle is relatively rapid in comparison with an isotonic contraction. As is shown in this study, the tension development in strict isometry is practically completed about 100 seconds after the start with 2.3×10^{-4} M ATP concentration. This applies even to thick bundles. An isotonic contraction, on the other hand, has not yet entirely reached a constant level 20 minutes after the start under similar conditions (EDMAN 1953). As mentioned earlier, the inactive parts in the fibre bundle, those not reached by ATP, must oppose the shortening of the active layer of the fibre bundle. If this braking has a viscous component, it would at least partly explain why the isometric contraction course is more rapid. The difference in the rate of contraction may also mean that the elementary process for shortening is appreciably slower than that for tension development. It is further possible that the diffusion of ATP into the fibres is made more difficult as the fibres shorten in an isotonic contraction, nor is it impossible that the ATP consumption in the already contracted layer of the fibre is different in isotonic and isometric contraction, as pointed out above. This would affect the time course for the penetration of ATP into the fibres.

ADP (PORTZEHL 1952, H. H. WEBER and PORTZEHL 1952) and inorganic phosphate (NEWBOLD and ROSE 1957), which are formed by the splitting of ATP, have a relaxing effect on the glycerol-extracted muscle fibres. The relaxing effect of ADP is manifest even at 2.5×10^{-4} M concentration at isometric registration (EDMAN, unpublished). Thus, it is conceivable that the contraction-inducing effect of ATP may be inhibited by the breakdown products accumulated in the fibres through the splitting of ATP. Attainment of a constant concentration profile for the breakdown products will always be slower than that for ATP. Therefore, if mechanisms of this sort play a role, the tension would rise to a maximum and then drop again. Since this does not happen the breakdown products do not seem to have any importance in the course of the contraction.

The diffusion of ATP into fibre bundles.

As shown in section 2 under Results, a comparison between the contraction course of fibre bundles of different thickness at the same ATP concentration can give information on how deeply ATP in effective concentration diffuses into the fibre bundle. The fact that the tension reaches a constant level for thinner bundles at a time when the tension is still increasing in thicker fibre bundles must mean that ATP in the thin bundles has reached the centre. It might be expected that the contraction curve would reach a constant level later and later with increasing thickness of the fibre bundle, up to the thickness at which ATP just reaches the central parts in effective concentration. Further increase in the thickness of the fibre bundle would not delay the attainment of a constant ATP concentration profile. From the experiments carried out along this line it can be established that the thicker the fibre bundle the later constant tension is reached, at least up to a thickness of 80–90 μ with an external ATP concentration of 2.3×10^{-4} M, as shown in figure 4. This must mean, accordingly, that ATP at a concentration of 2.3×10^{-4} M can penetrate 40 μ into a fibre bundle in effective concentration. Since the shape of the cross section differs from bundle to bundle and this shape affects the contraction curve, this method cannot be used for more than an approximate estimation of the diffusion range of ATP into the bundles.

There are two limiting possibilities for the diffusion of ATP into a fibre bundle:

- i. The fibre bundle may be regarded as a homogeneous body. The results obtained would mean in this case that the entire cross section area of the fibre bundle is reached by ATP to a depth of 40 μ .
- ii. The fibre bundle may be regarded as an inhomogeneous body with completely different diffusion conditions for ATP in the fibres and in the tissue between the fibres, *i. e.* the endomysium. Simply the fact that there is probably no breakdown of ATP in the endomysium must mean that this tissue comprises a more easily passable path for ATP than the fibres themselves. Due to the consumption of ATP in the fibres, however, the ATP will decrease in concentration during diffusion into the fibre bundle. Thus, a concentration gradient would arise with respect to ATP from the periphery in towards the centre both for the fibre bundle as

a whole and for each individual fibre within the ATP-containing part of the bundle. In sufficiently thick fibre bundles the central fibres would be entirely without ATP.

Which of the two possibilities presented is most probable? Assuming the entire fibre cross section as active, A. WEBER (1951) found a maximum tension development for single, glycerol-extracted psoas fibres of 4 kg/cm² at $5-8 \times 10^{-3}$ M ATP. According to HASSELBACH's (1952) investigations, however, ATP can penetrate only to a certain depth into the fibres, and therefore this value for the specific tension development must be underestimated. In the limiting case where the depth of diffusion is equal to the radius of the fibre MEYERHOF-SCHULZ' (1927) formula applies:

$$r = \sqrt{\frac{4 \cdot C \cdot D}{A}} \dots \dots \dots (1)$$

If, on the other hand, the depth of diffusion (d) is small in relation to the radius of the fibre, the following formula applies:

$$d = \sqrt{\frac{2 \cdot C \cdot D}{A}} \dots \dots \dots (2)$$

This is obtained from WARBURG's (1923) formula for determination of "Grenzschichtdicke". In both formulae C denotes the external concentration, D the diffusion coefficient in the fibre, and A the rate of splitting of ATP. If HASSELBACH's (1952) values for A (10^{-6} M/cm²/sec) and D (2×10^{-8} cm²/sec) are used, according to formulae (1) and (2) diffusion depths of 7.5 μ and 5.3 μ respectively are obtained with a concentration of 7×10^{-3} M, which lies in the middle of the interval used by A. WEBER. Since her fibres are about 50 μ in diameter, the true figure is probably closer to the lower limit. We will assume it to be 6 μ . With this diffusion depth the specific tension development of the active part of the fibre would be at least 9.4 kg/cm². For 2.3×10^{-4} M ATP at least half this value may be anticipated, *i. e.* about 4.7 kg/cm², in accordance with figure 6, which shows the relation between the ATP concentration and the tension in glycerol-extracted muscle fibre bundles (see discussion, page 250 for further details).

If, according to assumption (i) the entire cross section area of the fibre bundle is reached by ATP in to a depth of 40 μ , the tension development for the thinnest fibre bundles in figure 4 would correspond to about 1 kg/cm² and for the thickest to 0.3—

0.6 kg/cm². In calculating these values the cross section of the fibre bundle has been taken as rectangular. The values are considerably lower than the specific tension development that, according to the foregoing, may be expected with the ATP concentration used. This indicates that the active part of the cross section is overestimated. Assumption (ii) is therefore more probable, *i. e.* that ATP penetrates 40 μ into the fibre bundle via the endomysium, but can diffuse only a limited distance into the individual fibre. For calculation of the effective cross section area in this case it is assumed that the fibre bundle has a rectangular cross section and that the individual fibres are 40 μ thick, quadratic in their cross section and packed closely together in the fibre bundle. The depth of the diffusion into the fibre bundle obtained is thus assumed to correspond to the thickness of an individual fibre. With these assumptions the mean depth of diffusion for ATP into the individual fibre would amount to only about 1 μ for the total tension development obtained with 80—185 μ thick fibre bundles, to correspond to a specific tension development of 4.7 kg/cm². The values computed in this manner for the depth of diffusion into the individual fibre agree well with HASSELBACH's (1952) studies on the diffusion of ATP into thin sections of glycerol-extracted fibres. The values for the diffusion coefficient and the rate of splitting for ATP, as given by HASSELBACH, would, according to formulae (1) and (2) given earlier, permit ATP to diffuse approximately 1 μ into a glycerol-extracted fibre if the external concentration of ATP were 2.3×10^{-4} M as in the present investigation.

For determination of the rate of splitting and the diffusion coefficient for ATP HASSELBACH used 10 μ thick microtome sections of glycerol-extracted muscle fibres. It is probable that certain sections consequently contained endomysium also. Therefore, HASSELBACH's values cannot be assumed strictly to represent the diffusion conditions in the fibre itself. However, this source of error in HASSELBACH's determinations would not seem to be of fundamental importance in the present connexion.

The relation between final tension and the thickness of the fibre bundle.

Since ATP is able to diffuse only to a certain depth into a fibre bundle, the total tension development for thick fibre bundles may be expected to be proportional to the circumference and not

to the total cross section area. The results presented in figure 5 agree with this line of reasoning. As is evident from this figure the proportionality between total tension and circumference applies as well for fibre bundles that are thinner than $80\ \mu$ and which could consequently be reached by ATP in their central parts, as shown earlier. That there is direct proportionality between final tension and circumference even for thin bundles must mean that the ATP concentration in the central parts is relatively low so that, practically, the cross section area is active only in a peripheral layer of the fibre bundle. The group of experiments that represented extremely thick fibre bundles gave deviating results. This was probably due to technical difficulties in the performance of the experiments. The fibre bundle can be damaged at the point where it is tied to the thin platinum loops, and such damage must occur more easily with thick bundles.

The relation between final tension and ATP concentration.

The total isometric tension development of glycerol-extracted muscle fibre bundles increases with increasing ATP concentration, asymptotically approaching a maximum, as shown in figure 6. With 10^{-3} M ATP concentration the optimal tension seems practically to have been attained. RANNEY (1954 b), who used somewhat thinner fibre bundles of glycerol-extracted psoas from dogs, obtained optimal isometric tension with 6×10^{-4} M ATP. The value for maximum tension given in RANNEY's work is higher than that obtained in the present investigation. This may have been due to a difference in species. In studies of the contractility of glycerol-extracted cell models HOFFMAN-BERLING and H. H. WEBER (1953) found maximum shortening at about 2×10^{-3} M ATP concentration. For isotonic contraction of glycerol-extracted muscle fibre bundles (0.5—1.0 mm diameter) from rabbit psoas KOREY (1950) reported submaximum shortening even with ATP concentrations as high as 4×10^{-2} M. Raising the ATP above the optimal concentration gives a decrease in the tension development, as demonstrated by A. WEBER and H. H. WEBER (1951). According to these writers ATP begins to have such superoptimal effect at about 10^{-2} M concentration as shown in tests on the tension development of single glycerol-extracted rabbit psoas fibres.

The tension will increase with increasing ATP concentration until the concentration has become superoptimal in the outer-

most layer. The decrease in tension in the superficial layers will then start to compensate the increase in tension in the deeper layers. For this reason the diagram in figure 6 can only approximately reproduce the relation between specific tension (tension per unit of active cross section area) and ATP concentration. The increased tension with rising ATP concentration is due not only to higher specific tension but also to the deeper diffusion of ATP into the fibre bundle and the individual fibres and to the consequent activation of a greater portion of the cross section area. This results in underestimation to a certain degree of the specific tension development with low ATP concentrations as compared with the tension development with high ATP concentrations. It is evident, accordingly, from figure 6 that the specific tension development with 2.3×10^{-4} M must be at least 50 % of the possible maximum. This is of importance in the discussion on pages 246—248.

The rate of isometric contraction.

The rate of isometric contraction is the tension increase per unit of time. With fibre bundles of different dimensions, under otherwise equal conditions, at least the initial tension increase will be proportional to the length of the front over which ATP diffuses into the fibre. Consequently, an initial *absolute rate* may expediently be expressed in tension increase per circumference per unit of time, and thus it becomes independent of the geometry of the fibre bundle. In the continued contraction the rate becomes a complicated function of the geometry of the fibre bundle and the individual fibres, of the ATPase activity, the ATP concentration and several other variables. It is conceivable that fibre bundles, identical in all these respects, could show different degrees of contractility, *i. e.* maximum force development per unit of contractile substance, under the influence of special substances or for other reasons. Differences, in this respect would give rise to differences in the rate of contraction, both initially and in the continued course. In order to compensate for factors of this nature, it is possible to use the *relative rate*: the fraction of the final tension developed per unit of time. The relative contraction rate is independent of the absolute magnitude of the tension developed during the course of the contraction. The manner in which a variation in the ATP concentration influences the relative con-

traction rate of an isometric contraction is illustrated in figure 7. Inasmuch as it is not possible to know beforehand how a substance is going to modify the course of the contraction and thereby the shape of the contraction curve, it is necessary to compare the contraction curves as a whole, concerning as well the absolute as the relative rate of contraction. Only then can a complete picture be obtained of the effect of a substance on the contraction rate.

Latency.

There is a diffusion problem even before ATP has reached the surface of the fibre bundle. When the bundle is immersed in the ATP solution an unstirred layer (SCHULMAN and TEORELL 1938) will exist immediately surrounding the fibre bundle. Before any contraction can take place, this layer must be penetrated by ATP so that a certain concentration is attained in the periphery of the fibre bundle. Experimentally it is found that the contraction occurs only after a certain latent period following immersion in ATP, as shown in figure 3. The fact that the latency decreases with rising ATP concentration over the range possible to study with this recording device is what would be expected if ATP must diffuse through a passive fluid layer in order to reach the fibre bundle. For computation of the thickness of this boundary layer, taking the values obtained for the latent period as point of departure, the following formula may be used (KOHLEBAUSCH 1947):

$$c = c_0 \left[1 - \psi \left(\frac{z}{2 \sqrt{k \cdot t}} \right) \right] \dots \dots \dots (3),$$

where k is the diffusion coefficient, z the diffusion distance, *i. e.* approximately the thickness of the boundary layer, t the diffusion time, c the concentration at the inner surface of the boundary layer and c_0 the concentration in the bulk of the fluid. ψ represents the Gaussian integral of error. This equation can be solved for z . The diffusion time t may be considered approximately equal to the latency. ATP concentration 2×10^{-3} M is used for c , the concentration given by A. WEBER (1951) as the threshold value for obtaining tension development in glycerol-extracted psoas fibres. The diffusion coefficient for free diffusion of ATP in 0.2 M KCl decreases with rising ATP concentration according to HASSELBACH (1952). The lowest concentration used by HASSELBACH was about 8×10^{-4} M. If his values are extrapolated, a diffusion

coefficient of 5.5×10^{-6} cm²/sec is obtained for 6.0×10^{-4} M ATP. If we assume that this value also applies for lower concentrations, the latencies obtained for ATP concentrations up to 6.0×10^{-4} M correspond to a diffusion distance of 31–45 μ in accordance with the foregoing formula. The value thus computed agrees well with the thickness of the boundary layer at membrane and monolayer interfaces in water solution observed by SCHULMAN and TEORELL (1938). Accordingly, it is probable that the latency mainly is due to the fact that ATP must diffuse through an unstirred layer.

The inactive centre of the fibre bundle.

In higher degrees of isometry, as in these investigations, the inactive nucleus in the fibre bundle ought not to slow the contraction appreciably. It is possible, however, that part of the longitudinal extent of certain fibres lies in the ATP-containing outer layer and part in the ATP-free nucleus of the fibre bundle, *i. e.* that the fibres do not lie entirely parallel. In the contraction of such a fibre it is possible that a certain degree of compensatory stretching of the part in ATP-free medium takes place. Thus, for the fibres that are not in the outer, ATP-containing layer of the bundle throughout their entire length, defective isometry might occur during the contraction. The fibres are adherent to each other, however, which counteracts stretching of an individual fibre. Moreover, as far as can be determined by microscopic study of the 10 mm long fibre bundles, the fibres run parallel. However, the possibility of a certain deviation from isometry because of deficient parallelism of the fibres cannot be completely precluded in the contraction of thick fibre bundles.

Summary.

A new technique for strictly isometric recording of glycerol-extracted muscle fibre bundles is described. The apparatus works on the principle of a torsion balance.

By comparing contractions of fibre bundles of different thickness from rabbit psoas conclusions can be drawn about the diffusion depth for ATP into the bundle. ATP in a concentration of 2.3×10^{-4} M penetrates about 40 μ into a fibre bundle, probably via the endomysium, the diffusion into a single fibre amounting to only about 1 μ .

The final isometric tension is proportional to the circumference of the fibre bundle, not to the cross section area.

The magnitude of the final isometric tension and the rate of the isometric contraction have been studied as functions of the ATP concentration.

The latency between the moment of immersion of the fibre bundle into the ATP solution and the start of the contraction is compatible with the assumption of an unstirred fluid layer of 30–45 μ around the fibre bundle.

Possible factors influencing the course of isometric contraction of glycerol-extracted muscle fibre bundles are discussed.

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The Effect of Iproniazid on the Blood Pressure Responses in Dogs to Histamine Injected into the Renal Artery.

By

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In two previous investigations it was found that histaminase inhibitors potentiated the depressor responses to histamine injected into the renal artery (LINDELL and WESTLING 1956 and LINDELL 1957). Evidence was obtained that the potentiation was due to inhibition of the histaminase in the kidney, and that this histaminase was one of the factors responsible for the renal removal of injected histamine. In both studies, however, even after the administration of histaminase inhibitors in large amounts the kidneys retained a considerable capacity to remove histamine. It seems possible that this remaining capacity to remove injected histamine was due to SCHAYER's "histamine metabolizing enzyme II" (SCHAYER 1953). Changes produced by iproniazid (Marsilid) in the pattern of the urinary metabolites of histamine led SCHAYER, KENNEDY and SMILEY (1953) to the conclusion that iproniazid inhibits the "histamine metabolizing enzyme II".

In the present investigation the influence of iproniazid on the renal removal of injected histamine was studied in dogs with the technique used by LINDELL (1957). Histamine was injected into the renal artery and the depressor responses compared to those after intravenous injections of histamine. Iproniazid potentiated the depressor responses to histamine given into the renal artery. However, if iproniazid was given to dogs which had already received a potentiating dose of aminoguanidine no further potentiation occurred.

Methods.

Five dogs weighing 9–21 kg were used. The experiments were performed in the same way as those recently described by LINDELL (1957) and, therefore, a brief description only will be given here. The dogs were anaesthetized with sodium pentobarbitone (Nembutal Abbot) by vein. A polythene catheter was inserted into a renal artery from a femoral artery using the technique of LINDELL and OLIN (1957). Another polythene tube with the same dimensions was inserted into the inferior caval vein. Histamine was injected through these tubes with motordriven syringes. The blood pressure responses were recorded with a mercury manometer connected to a carotid artery. The inhibitors were infused through other polythene tubes inserted into suitable veins in the legs. At the end of the experiment the kidneys were removed. Crude histaminase preparations made from these kidneys were incubated with histamine and cadaverine and the oxygen uptake measured with the Warburg manometric technique.

Drugs: Histamine acid phosphate. The injected doses are given in μg per kg b. wt. per minute. Aminoguanidine sulphate and iproniazid were used as inhibitors.

Results.

The effect of iproniazid on the blood pressure responses to histamine injected into the renal artery.

The effect of iproniazid alone on the depressor responses to histamine injected into the renal artery and into the caval vein were studied in two dogs (201 and 202). The course of these experiments is best illustrated by the blood pressure record of exp. 202 (fig. 1). After the injection of some preliminary doses of histamine into the renal artery and into the caval vein, two approximately equiactive doses were given repeatedly, injections into the renal artery alternating with injections into the caval vein. 10–15 times more histamine had to be injected into the renal artery than into the caval vein to obtain equal depressor responses. This agrees well with the relations found earlier (LINDELL 1957). Iproniazid was then infused by vein at a rate so adjusted that it had no effects of its own on the blood pressure. The total amount of iproniazid was 130 mg/kg in both experiments. The infusion of iproniazid caused an augmentation of the depressor responses to histamine given into the renal artery, whereas the responses to histamine given by vein remained unchanged. The greatest potentiation obtained was such that the relations between equiactive

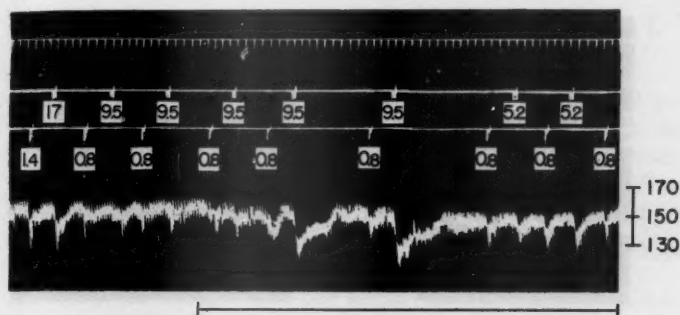


Fig. 1. Dog 202. The effect of iproniazid alone on the depressor responses to histamine injected into the renal artery and into the caval vein.

From above: time in 30 seconds, injections into the renal artery, injections into the caval vein (doses of histamine are given in $\mu\text{g/kg}$ b. wt./min.) blood pressure in a carotid artery. — indicates the infusion of iproniazid, 130 mg/kg.

doses of histamine in the renal artery and in the caval vein were approximately 6 to 1.

Effect of iproniazid when given after aminoguanidine, on the depressor responses to histamine injected into the renal artery.

In three dogs (203, 204 and 205) histamine was injected into the renal artery and the caval vein in the same way as in exp. 201 and 202. Aminoguanidine, 13 mg/kg was then infused during a period of 10 minutes. This potentiated the responses to histamine given into the renal artery so that the relations between equiactive doses of histamine injected into the renal artery and into the caval vein were approximately 4 to 1. In exp. 203 and 204 the infusion of aminoguanidine was continued throughout the experiment at the rate of 0.5 mg per minute. The depressor responses to different doses of histamine injected into the renal artery and into the caval vein were then recorded as shown in fig. 2, I. After this, two approximately equiactive doses, one in the renal artery and one in the caval vein, were given repeatedly while iproniazid was infused by vein. The total amount of iproniazid given was 111, 140 and 125 mg/kg in dogs 203, 204 and 205, respectively. These amounts did not change the relations between equiactive doses of histamine injected into the renal artery and into the caval vein. The experiments were then

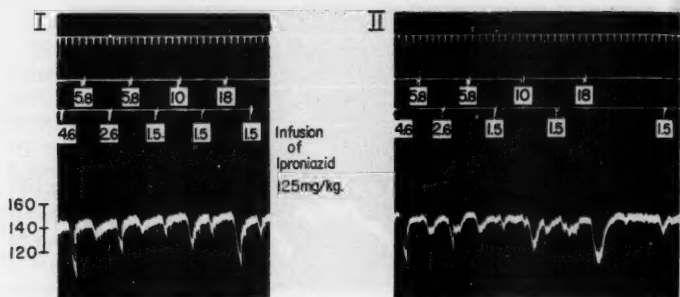


Fig. 2. Dog 205. The effect of iproniazid on the depressor responses to histamine injected into the renal artery and the caval vein of a dog which had already received aminoguanidine (13 mg/kg).

From above: time in 30 seconds, injections into the renal artery, injections into the caval vein (doses of histamine are given in $\mu\text{g/kg}$ b. wt./min.) blood pressure in a carotid artery. Between I and II iproniazid (125 mg/kg) was infused during a time of 50 minutes.

completed with a repetition of the different doses of histamine given before the administration of iproniazid (fig. 2, II). In no instance was an increase in the depressor responses to histamine observed after the administration of iproniazid to these three dogs which had previously received aminoguanidine.

Effect of the injected iproniazid on the oxidation of histamine and cadaverine by crude histaminase preparations from the kidneys removed at the end of the experiments.

The kidneys from the two dogs injected with iproniazid alone (130 mg/kg) were removed at the end of the experiments. Crude histaminase preparations were made from these kidneys as described by LINDELL (1957). The addition of histamine or cadaverine to these preparations caused no oxygen uptake as measured with the Warburg manometric technique. When such enzyme preparations from dogs not given inhibitors were incubated with histamine and cadaverine the oxygen uptake averaged respectively 40 and 90 microliters per gram kidney per hour (LINDELL 1957).

Discussion.

In the present experiments it was found that iproniazid potentiated the depressor responses to histamine injected into the renal artery. The potentiation was of approximately the same order of magnitude as that obtained with aminoguanidine. This histaminase inhibitor in a dose of 10 mg/kg has little or no effect on "the histamine metabolizing enzyme II" in cats (SCHAYER, KENNEDY and SMILEY 1953).

When given to dogs which had already received aminoguanidine in a potentiating dose, iproniazid did not produce any further potentiation. This indicates that, as far as the potentiation of the responses to histamine injected into the renal artery is concerned, iproniazid and aminoguanidine act through a common mechanism. Available evidence makes it likely that aminoguanidine acts through inhibition of the histaminase in the kidney (LINDELL and WESTLING 1956 and LINDELL 1957). SCHAYER (1953) found that iproniazid inhibited the formation of the compound of peak 1 in chromatograms of urine from mice injected with C^{14} histamine. This peak represents an end product of the action of diamine oxidase (histaminase). Thus, iproniazid may inhibit the histaminase in the living animal. The view that iproniazid acted through the inhibition of histaminase in the present experiments may also receive some support from the observation that the oxidation, not only of histamine but also of cadaverine, by the enzyme preparations from the kidneys removed after injection of iproniazid was inhibited.

The amounts of iproniazid used in the present study were of the same order of magnitude as those which inhibited the "histamine metabolizing enzyme II" in cats (SCHAYER, KENNEDY and SMILEY 1953). However, the fact that no further potentiation occurred, when iproniazid was given to dogs which had already received aminoguanidine, does not necessarily mean that there is no "histamine metabolizing enzyme II" in the dog's kidney. According to SCHAYER and KARJALA (1956) the "histamine metabolizing enzyme II" is a composite of a methylating and an oxidative enzyme. The first step in this pathway for the metabolism of histamine is the formation of 1-methyl-4-(β -aminoethyl)-imidazole, (methylhistamine). This step is not inhibited by 1-isobutyl-2-isonicotinylhydrazine (IBINH). The methylhistamine is

then oxidized to 1-methylimidazole-4-acetic acid and this oxidation can be inhibited by IBINH. In the experience of the present author this methylhistamine is about 300 times less active than histamine in causing a depressor response when given by vein to dogs. Thus the methylation is almost the same as inactivation. It is likely that iproniazid acts in the same way as IBINH, *i. e.* by inhibiting the oxidation but not the methylation. Then, even if the canine kidney contains the "histamine metabolizing enzyme II" iproniazid should not potentiate the depressor responses to histamine injected into the renal artery of dogs which have already been given aminoguanidine in a dose sufficient to inhibit completely the histaminase in the kidney.

Summary.

1. Iproniazid potentiated the depressor responses in dogs to histamine injected into the renal artery.

2. Some evidence was obtained that this potentiation was due to an inhibition of the histaminase in the kidney rather than to any interference with the action of a "histamine metabolizing enzyme II".

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Myocardial Oxygen Consumption and Coronary Blood Flow during Potassium-Induced Cardiac Arrest and during Ventricular Fibrillation.¹

By

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With the technical assistance of Eleanor Gotz, Philip E. Waithe and Louis Freni.

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Cardiac arrest, induced by injection of potassium (MELROSE, DREYER, BENTALL and BAKER 1955), has recently become an adjunct to cardiac surgery. During arrest the inflow into the coronary arteries from the pump-oxygenator has been blocked for periods of up to one-half hour, apparently on the assumption that the myocardium would not need any substantial amount of oxygen during this time. In the present study the oxygen consumption of the heart in arrest was determined while the coronary vessels were perfused at a constant pressure from a donor dog. The values were compared with those obtained during ventricular fibrillation and cardiac work in the same dog. The data also provide information on the role of metabolic versus mechanical factors in the regulation of coronary blood flow.

Method.

The experiment dogs were premedicated with morphine sulfate (1.8—2.4 mg/kg) and anesthetized with chloralose (50—65 mg/kg) and urethane (500—650 mg/kg). After thoracotomy the animals were ventilated with a Starling pump. The donor dogs were anesthetized

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with chloralose and urethane and also ventilated with a Starling pump. After necessary cannulations the experiment and donor dogs were connected as follows. The left main coronary artery, cannulated with a GREGG cannula, was perfused from a carotid artery of the donor dog. During arrest and fibrillation the aorta was perfused from the donor dog's femoral arteries. Adequate return of blood from the experiment dog was directed from the right atrium to the donor dog's femoral veins.

Coronary blood flow was measured with a rotameter (SHIPLEY and WILSON 1951). It was attempted to keep the coronary perfusion pressure (measured between flowmeter and coronary cannula) at a constant level and above 60 mm Hg. Aortic and/or left ventricular pressures and left atrial pressure were recorded with strain gage manometers. In the later experiments a Colson optical densitometer (GILFORD, GREGG, SHADLE, FERGUSON and MARZETTA 1953) was used for observing variations in coronary sinus oxygen saturation. These values and a lead II electrocardiogram were recorded on direct-writing oscillographs. Coronary sinus samples were obtained from a Goodale-Lubin (U. S. Catheter Co.) or a polyethylene catheter, introduced far into the coronary sinus via the right jugular vein. Arterial and venous blood samples were analyzed for oxygen content by the method of VAN SLYKE and NEILL. Myocardial oxygen consumption was calculated as the product of coronary blood flow and coronary a-v oxygen difference.

Cardiac arrest was induced by rapid injection of 10–20 ml of a 5 per cent potassium citrate solution into the coronary artery tubing and was maintained for a desired period of time by a continuous slow infusion (0.25–2.0 ml/min) of the same solution. Ventricular fibrillation was induced by an electric shock of 60-cycle alternating current. Defibrillation was done with a more intense shock of alternating current. In one experiment acetylcholine was given in an attempt to produce asystole; complete asystole was however not produced with that procedure.

All blood oxygen samples were taken after a steady state had been reached, as indicated by the pressure, coronary flow and densitometer readings.

Results.

Figure 1 shows the time sequence and results of one of the experiments. "Control values" were usually obtained before and after each period of arrest or fibrillation. In two experiments two arrest periods more than one-half hour apart were studied. The arrest periods were 17 to 37 minutes.

It is seen in Figure 1 that the myocardial oxygen consumption was much lower in arrest than during control work and fibrillation. In this animal the reduction of oxygen consumption was accompanied by a concomitant decrease in both coronary flow

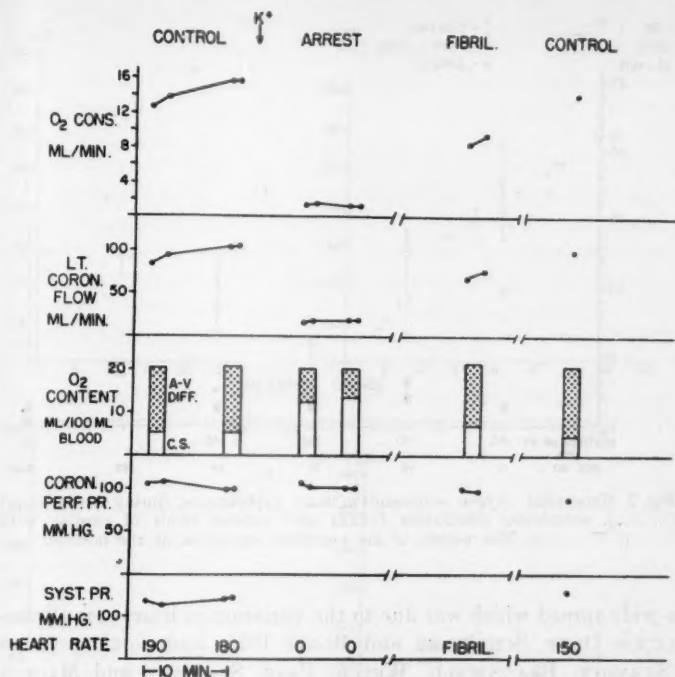


Fig. 1. Changes in myocardial oxygen consumption, coronary blood flow and coronary a-v oxygen difference during arrest and fibrillation. Dog no. 26. Heart weight 145 g, ventricular weight 130 g. "Syst. Pr." denotes ventricular systolic pressure during cardiac work. The indicated breaks in the time scale were one-half hour and one-quarter hour, respectively.

and a-v oxygen difference. The coronary flow during arrest fell despite the almost constant perfusion pressure.

Oxygen consumption values: Figure 2 shows the oxygen consumption values from six experiments during arrest, fibrillation and cardiac work. In all but one dog the oxygen consumption during arrest was less than 3.5 ml/minute. In one dog (no. 25) the arrest oxygen consumption was markedly higher. The heart weight was within the range of the others. In this dog it was exceptionally difficult to maintain electrical and mechanical inactivity of the heart, and potassium citrate was given in such a large amount that the donor dog's heart fibrillated.

During fibrillation the oxygen consumption was 4-9 times higher than during arrest. The values during cardiac work showed

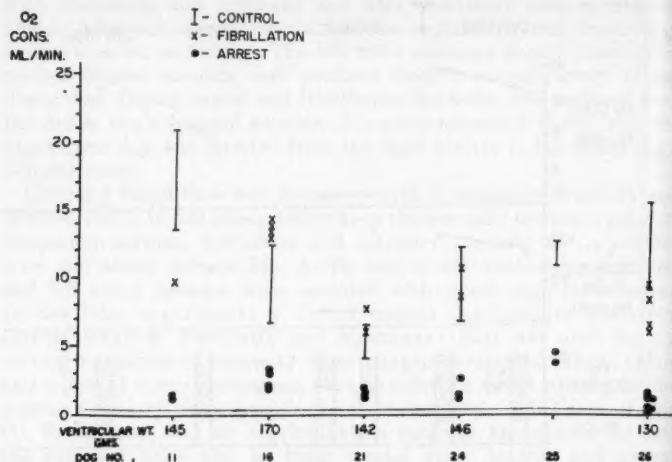


Fig. 2. Myocardial oxygen consumption in six experiments, during cardiac arrest (.....), ventricular fibrillation (xxxx) and various levels of cardiac work (/—/). The weight of the ventricles are given at the bottom.

a wide spread which was due to the variation in heart rate (BERGLUND, DUFF, SCHREINER and BORST 1957) and aortic pressure (SARNOFF, BRAUNWALD, WELCH, CASE, STAINSBY and MACRUZ 1957).

Coronary blood flow: In all experiments there was at the onset of arrest an immediate rise in coronary blood flow but within five minutes the coronary blood flow had stabilized at a lower level. In four of the experiments the coronary flow was much lower during arrest than during cardiac work and fibrillation. It is seen in Figure 3 that in these dogs coronary flow increased linearly with oxygen consumption, despite the constant coronary perfusion pressure. In one dog (no. 16) the coronary flow was directly proportional to the oxygen consumption, and the coronary a-v difference varied only between 8.7 and 12.4 ml/100 ml blood throughout the experiment. In the other experiments shown in Figure 3, the correlation was not as good. The a-v difference in dog no. 11, for example, varied between 2.2 and 13.9 ml/100 ml blood.

In the other two dogs, not shown in Figure 3, the relation between coronary flow and oxygen consumption was less striking.

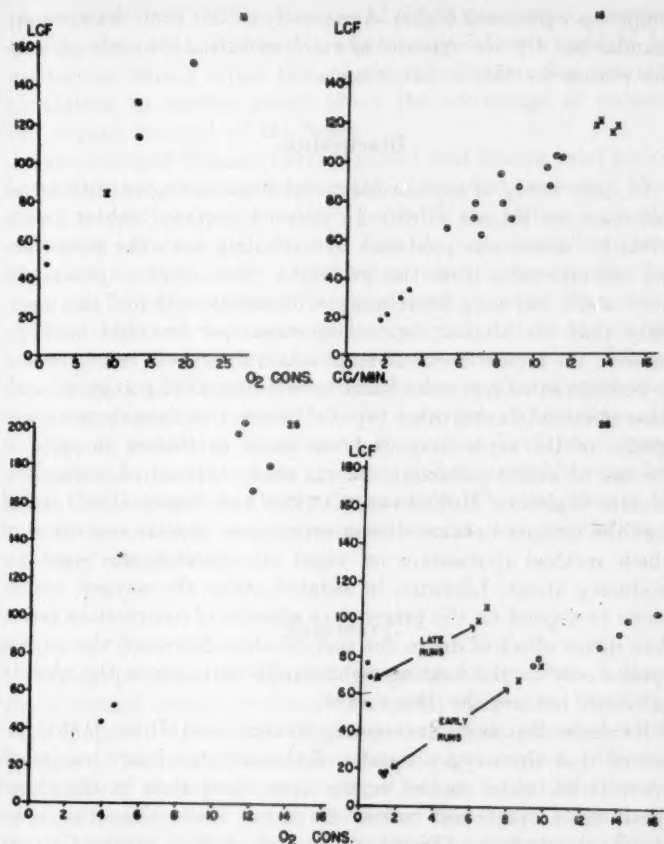


Fig. 3. Relation between coronary flow and myocardial oxygen consumption during arrest (....), fibrillation (x x x) and cardiac work (o o o). Experiment numbers are given in the respective upper right hand corners.

In these dogs the coronary vessels were apparently less active than in the other dogs.

One finding, shown in experiment no. 26 in Figure 3, deserves more comment. During the early runs the coronary flows at given oxygen consumptions were relatively low. In later runs of arrest and fibrillation, one-half to one and one-half hours after the last of the "early runs", the coronary flows at given oxygen con-

sumptions were much higher. Apparently at this time the coronary vascular bed did not constrict as much as during the early periods. The reason for this is not known.

Discussion.

An early study of myocardial metabolism during cardiac arrest was done by ROHDE (1910). In isolated perfused rabbit hearts reversible arrest was produced by excluding both the potassium and calcium salts from the perfusate. The oxygen uptake was 0.021 and 0.027 ml/g heart/minute. ROHDE mentioned the possibility that the altered ionic composition *per se* could have influenced the metabolism and emphasized that these values cannot be considered to represent basal metabolism until compared with those obtained during other types of arrest. Our data show oxygen uptake of the same magnitude as those of ROHDE in spite of the use of excess potassium in our study instead of potassium-calcium depletion. McKEEVER, CANNEY and GREGG (1957) found that the oxygen uptakes during arrest were similar regardless of which method (potassium or vagal stimulation) was used for producing arrest. Likewise, in isolated atriae the oxygen uptake seems to depend on the presence or absence of contractions rather than direct effect of drugs, for acetylcholine decreased the oxygen uptake only in the beating right auricle but not in the already quiescent left auricle (HOLZ 1938).

Recently BEUREN, SKIBINSKY, SPARKS and BING (1957) reported that the oxygen uptake of the arrested heart was much lower (1/10) when studied in the open chest than in the closed chest; their open-chest values are much lower than the open-chest values of the present study and of McKEEVER, CANNEY and GREGG (1957). Their results cannot be fully evaluated before a more complete description of their experiments has been published.

The difference between oxygen consumption values obtained during arrest and fibrillation is consistent with findings from GREGG's laboratory (McKEEVER et al. 1957) and others (SENNING 1952, JARDETZKY, GREEN and LORBER 1956). This is not surprising, for although the heart is not performing any external work, random areas of the heart muscle are contracting at a rapid rate. The situation is analogous to the increase in oxygen consumption with increased heart rate at a given work level

(BERGLUND et al., 1957). Because of the lower oxygen consumption during arrest, obstruction of coronary inflow should be less deleterious during arrest than during fibrillation. Conversion of fibrillation to cardiac arrest offers the advantage of reducing the oxygen demand of the heart.

SABISTON and GREGG (1957) reported that during brief periods of cardiac arrest induced by vagal stimulation, coronary blood flow increased above the "control levels" if the perfusion pressure was artificially maintained; our data do not contradict theirs. However, in four of our experiments, when a steady state had been reached, the coronary flow was lower in arrest than during cardiac work and was more or less proportional to the oxygen consumption. The immediate increase in coronary flow observed by SABISTON and GREGG was evidently due to altered mechanical conditions, namely removal of the vascular compression. The subsequent reduction of coronary flow must have been caused by increased tone of the coronary vascular bed, probably secondary to the lower oxygen need of the myocardium. This emphasizes the role of myocardial oxygen consumption in the regulation of coronary blood flow.

Summary.

During perfusion of the left coronary artery from a donor dog's carotid artery, cardiac arrest and ventricular fibrillation were induced. Myocardial oxygen consumption, obtained as the product of coronary flow and coronary a-v oxygen difference, was reduced during arrest to values below 3.5 ml/minute in 5 of the 6 dogs studied. During fibrillation the oxygen consumption was 4—9 times as high as during arrest. The importance of these findings for cardiac surgery is emphasized. The role of myocardial oxygen consumption in determining coronary flow is discussed.

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On the Biochemistry and Pharmacology of Sodium Monothiophosphate.

By

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During a systematic survey of different sulfur containing compounds with respect to their ability to act as protective agents against radiation injury also sodium monothiophosphate was investigated (CLEMEDSON, HOLMBERG, NELSON and SÖRBO 1956). It was found to be inactive, but in that connection some of its biochemical and pharmacological properties were studied. The compound was previously shown by BINKLEY (1949) to be hydrolyzed to phosphate in the presence of tissue preparations, the other hydrolysis product presumably being hydrogen sulfide. Under aerobic conditions the monothiophosphate was oxidized to a compound suggested by BINKLEY to be thiosulfate.

The first part of this paper deals with the biochemical transformation of thiophosphate and its effect on phosphorylation systems. The second part is concerned with the general pharmacological properties of thiophosphate, compared with those of hydrogen sulfide.

Materials.

Trisodium monothiophosphate was synthesized according to WURTZ (1847). When injected, it was used as a 50 per cent solution brought to neutrality with HCl. Crystalline glyceraldehyde phosphate dehydrogenase (triose phosphate dehydrogenase) from muscle and its substrate in form of a mixture of glyceraldehyde phosphate and dihydroxy-

acetone phosphate) were obtained from C. F. Boehringer and Soehne GMBH. Diphosphopyridine nucleotide (DPN) was a gift from Ing. Å. ÅKESSON. Other compounds were commercial products of analytical purity.

Hydrogen sulfide was injected as $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in a 2.4 per cent solution, corresponding to 2.4 mg H_2S per ml.

Methods.

Biochemical methods.

The oxidation of thiophosphate was studied with a conventional Warburg manometer technique. The experiments were carried out at 37°C in air and the test system contained liver homogenate, 0.01 M thiophosphate and 0.1 M Tris buffer in a final volume of 3 ml and at a pH of 7.8. Thiosulfate was determined in the system with a recently developed method (SÖRBO 1957 a), based on the conversion of thiosulfate to thiocyanate with cupric ions and cyanides. The thiocyanate was then determined colorimetrically with ferric ions. As thiophosphate also was converted to thiocyanate under these conditions, it had to be removed prior to analysis. This was accomplished through addition of 0.5 ml of 1 M cadmium acetate and centrifugation, and thiosulfate was then determined in the supernatant as previously described (SÖRBO 1957 b).

The ability of thiophosphate to replace phosphate in the glyceraldehyde phosphate dehydrogenase reaction was studied spectrophotometrically at $340\text{ m}\mu$ in a test system, based on the observations of WARBURG and CHRISTIAN (1939). Thiophosphate as a substrate for rhodanese (in form of a rat liver homogenate) was tested in a system, similar to that described by SÖRBO (1953). The desired pH of the system (8.6), was in the case of thiophosphate obtained by addition of HCl. As thiophosphate gave a strong red colour with ferric ions, it was removed (and the enzymic reaction stopped) by addition of cadmium acetate, and thiocyanate was determined as usual in the supernatant. A system, containing thiosulfate, was given the same treatment.

Rat liver homogenates were prepared with a Bühler blender.

Pharmacological methods.

Acute toxicity. The acute toxicity of the compound was tested by intraperitoneal injection in albino mice weighing about 20 grams. LD_{50} was calculated according to the method of MILLER and TAINTER (1944).

Experiments in the anaesthetized cat. The cats were anaesthetized with sodium pentobarbital (30 mg per kg body weight) administered intraperitoneally. The right common carotid artery was cannulated and the blood pressure recorded on a smoked drum. The respiration was recorded by means of a pneumograph. The anterior tibial muscle was used for investigating the effects on neuromuscular transmission. The stimulation of its nerve was supramaximal and repeated every 6 sec.

To test for the occurrence of hydrogen sulfide in the expired air, a three-way valve was connected to the tracheal cannula and a filter paper soaked with lead acetate was held before the outlet of air. The substances were injected into the femoral vein.

Results.

Biochemistry.

When thiophosphate was shaken with rat liver homogenate in air, thiosulfate was identified as one of the reaction products. Thus 1 ml of a 9 per cent liver homogenate gave after 1 hour in the previously described system 1.78 μ moles of thiosulfate, a conversion of about 6 per cent. No formation of thiosulfate was obtained when the liver homogenate had been heated to 100° C for 2 min. As separate experiments demonstrated that hydrogen sulfide was formed from thiosulfate, when the latter was incubated with liver (the hydrogen sulfide was identified as cadmium sulfide, lead sulfide and with the methylene blue reaction), the formation of thiosulfate from thiophosphate can be explained by a two step mechanism. Thiophosphate is first hydrolyzed to hydrogen sulfide by a heat labile system in the liver (BINKLEY 1949 and the present investigation) and the following oxidation of hydrogen sulfide to thiosulfate is then a nonenzymic heme catalyzed reaction (SÖRBO 1956).

The ability of thiophosphate to replace phosphate in certain enzymic reactions was also investigated. Interesting results were obtained with triose phosphate dehydrogenase (Fig. 1). It is evident that addition of thiophosphate to a mixture of glyceraldehyde phosphate, DPN and enzyme gives a reduction of DPN in the same way as an addition of phosphate does. However, no definite equilibrium value is obtained with thiophosphate in contrast to that with phosphate. A similar, but more pronounced behaviour is obtained with arsenate (Fig. 1). This has in the case of arsenate been interpreted as a formation of an unstable arsenate ester, which rapidly decomposes, thus changing the equilibrium. Probably the same explanation is valid for thiophosphate, and consequently no attempts were made to isolate the intermediate reaction product. The observations made with triose phosphate dehydrogenase made it likely that thiophosphate should be able to "uncouple" oxidative phosphorylation. Attempts were made to demonstrate this "uncoupling" effect, using rat liver mitochondria as the phosphorylating system. From the effect

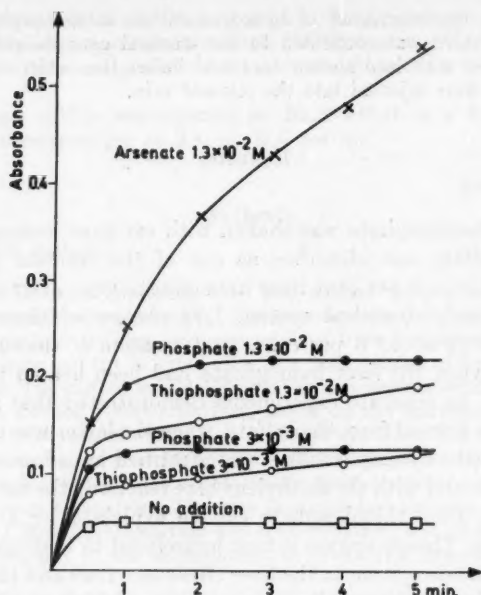


Fig. 1. Thiophosphate and triose phosphate dehydrogenase. The test system, of final volume 3.0 ml and pH 8.3, contained 0.024 M pyrophosphate, $1 \cdot 10^{-4} M$ DPN, 20 μg of enzyme and additions as indicated. The reaction was started by addition of 0.04 ml of 0.02 M glyceraldehyde phosphate and followed at 340 $m\mu$ in a Beckman spectrophotometer.

on oxygen consumption indications were also obtained for such an "uncoupling" but no clear-cut effects of thiophosphate could in fact be demonstrated, as rat liver mitochondria were found to hydrolyze thiophosphate rapidly to hydrogen sulfide and the effect of hydrogen sulfide on oxidative phosphorylation is not yet known.

It was mentioned previously, that thiophosphate behaved as thiosulfate in its reaction with cyanide and cupric ions. The possibility was now considered that thiophosphate could replace thiosulfate in the rhodanese reaction, but thiophosphate was found to be completely inactive in this respect.

Pharmacology.

Symptoms and toxicity. After intraperitoneal injection of lethal doses of sodium monothiophosphate in unanaesthetized mice, it usually took some minutes before any symptoms could be ob-

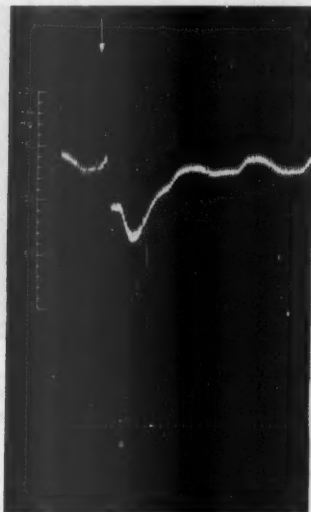


Fig. 2. Effect of sodium monothiophosphate on respiration, blood pressure and neuromuscular transmission in the cat. Record of respiration, carotid arterial blood pressure and contraction of anterior tibial muscle to supramaximal stimulation of the nerve at 10 per minute. Time at 60 sec. intervals. At arrow intravenous injection of 400 mg sodium monothiophosphate per kg body weight.

served. There were slight convulsions, and the respiration was slow and deep. Death seemed to be due to respiratory failure. The LD_{50} -dose was found to be 950 mg per kg body weight.

Experiments in the anaesthetized cat. After intravenous injection in the anaesthetized cat sodium monothiophosphate, in doses exceeding 200 mg per kg body weight, caused depression of the blood pressure. This response was immediate in onset but not of the same brief duration as *e. g.* acetylcholine evokes (Fig. 2). These low doses of sodium monothiophosphate also resulted in depression of the respiration, but this effect was not pronounced (Fig. 2). Doses exceeding 1,500 mg per kg body weight usually resulted in apnea, prolonged depression of the blood pressure and stand-still of the heart (Fig. 3). These responses were not changed by vagotomy or atropinization. Even at lethal doses the neuromuscular transmission was unaltered. Cumulative effects could not be demonstrated. Already after doses exceeding 100 mg per kg body weight the characteristic odour of hydrogen sulfide could



Fig. 3. Effect of sodium monothiophosphate on respiration and blood pressure in the cat. Record of respiration and carotid arterial blood pressure. Time at 60 sec. intervals. At arrow intravenous injection of 1,500 mg sodium monothiophosphate per kg body weight.

be felt, and the presence of hydrogen sulfide in the expired air could be demonstrated with the aid of the lead acetate paper after doses exceeding 400 mg per kg body weight. It was not possible to demonstrate sulphaemoglobin in the blood. After intravenous injection in the anaesthetized cat hydrogen sulfide (*cf. e. g.*

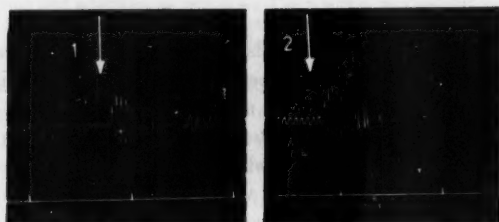


Fig. 4. Effect of hydrogen sulfide on the respiration in the cat. Time at 60 sec. intervals. At 1 intravenous injection of 0.4 mg hydrogen sulfide per kg body weight. At 2 intravenous injection of 2.0 mg hydrogen sulfide per kg body weight. Time between injections 20 minutes.

HAGGARD and HENDERSON 1922 and KMIETOWICZ 1931), already in doses exceeding 0.1 mg per kg body weight, caused pronounced hyperpnea followed by a short period of apnea (Fig. 4). These low doses also resulted in moderate elevation of the blood pressure. High doses, *i. e.* about 2 mg per kg body weight, also resulted in hyperpnea, but the apnea was prolonged and accompanied by

depression of the blood pressure and death. Hydrogen sulfide had no cumulative effects, and the responses were not changed by vagotomia or atropinization.

Discussion.

The *in vitro* experiments have demonstrated that thiophosphate is transformed to hydrogen sulfide, and this compound is evidently also formed *in vivo*, as it could be demonstrated in the expired air. However, the thiophosphate had an astonishingly low toxicity as compared with hydrogen sulfide. Furthermore, the pharmacological effects of thiophosphate and hydrogen sulfide do not support the assumption that the toxicity of thiophosphate is due to the formation of hydrogen sulfide. For instance, hydrogen sulfide in contrast to thiophosphate gives a pronounced hyperpnea.

An other possibility considered was that the effects of thiophosphate could be ascribed to an uncoupling of the oxidative phosphorylation. However, data obtained from the literature do not support this interpretation, as for instance dinitrophenol, a well-known uncoupler, has a pronounced stimulating effect on respiration* (MAGNE, MAYER and PLANTEFOL 1932, CUTTING, MEHRTENS and TAINTER 1933).

From the present experiments, therefore, no definite conclusions can be drawn as to the mechanism of the pharmacological action of the thiophosphate.

Summary.

1. Monothiophosphate is oxidized to thiosulphate in the presence of liver tissue. The mechanism of this reaction has been elucidated.
2. Thiophosphate can replace phosphate in the triose phosphate dehydrogenase reaction. However, an unstable intermediate is formed.
3. The LD_{50} -dose of the compound is 950 mg per kg body weight as determined by intraperitoneal injection in unanaesthetized mice.
4. Intravenous injection in the anaesthetized cat resulted in depression of respiration and blood pressure. No cumulative effects could be demonstrated. The neuromuscular transmission was unaffected.
5. The pharmacological effects of thiophosphate has been compared with those of hydrogen sulfide and found to be different.

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Pronounced Hypothermia Elicited by Prolonged Stimulation of the "Heat Loss Centre" in Unanaesthetized Goats.

By

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The experiments of MAGOUN, HARRISON, BROBECK and RANSON (1938) showing that local heating of the anterior hypothalamus and parts of the preoptic area mobilizes various physiological defenses against hyperthermia, demonstrate the presence of a "heat loss centre" within this part of the brain stem. The localization of a thermosensitive area to the region between the anterior commissure and the optic chiasma is further indicated by the work of ELIASSON and STRÖM (1950) and of v. EULER (1950). Weak electrical stimulation within this area causes polypneic panting, cutaneous vasodilatation and an inhibition of physiologically induced shivering in the unanaesthetized goat and repeated short periods of such stimulation in goats placed in a cold environment were found to lower the rectal temperature of the animals 1 to 1.5° C (ANDERSSON, GRANT and LARSSON 1956). It therefore seemed to be of interest to investigate whether similar but prolonged stimulation in a cold environment would cause a further fall of the body temperature of the animals, or, at any point, an increasing activity of structural elements concerned with the defense against cold would become strong enough to suppress completely the effects of the artificially induced activity of the "heat loss centre".

Methods.

Six adult female goats were used for these experiments. For electrical stimulation HESS' technique (1932, 1949) was used. The parameters of stimulation were 0.6 to 2.5 V and 50 pulses per second. Pulses were of 7 msec duration, slightly damped square waves with the D C component removed. The stimulations were made unilaterally.

A low environmental temperature was obtained by placing the animals in a freezing room where the temperature was kept fairly constant at -6 to -7°C . The rectal temperature and the ear surface temperature were in some experiments continuously recorded by help of thermocouples connected to an "Ellab" thermometer. In other experiments the rectal temperature was measured every 15th minute by help of a mercury thermometer placed at a certain depth of the rectum for 2 minutes.

Blood glucose was determined by the method of FOLIN and WU (1930). *Histological methods.* After the animals had been killed their heads were perfused first with Ringer's solution and then with Bodian's fluid and the brains were fixed in the latter and embedded in celloidin. Serial transverse sections, 100 microns thick, were made through that part of the brain which had been the site of the electrodes, and the sections were as much as possible directed parallel to the electrode tracks. The sections were stained with toluidin blue.

Results.

A. The exposure of normal goats to cold.

In order to compare the effect of low environmental temperature on normal goats to that on goats during continuous stimulation of the "heat loss centre", two intact animals were taken from room temperature (19°C) into -6°C and were kept there for three hours. Shivering appeared after about 5 minutes, gradually became more vigorous and after about 20 minutes reached a degree of intensity which remained the same during the rest of the time the animals were kept in the cold environment. The rectal temperature of the animals rose during the first 20 minutes of cold exposure about 1°C and then stayed at this level until the end of the experiment (Fig. 1, rect. control). The three hours these animals spent in the cold environment were not followed by any significant change of initial blood glucose level.

B. The effect of continuous stimulation of the "heat loss centre" in an environmental temperature of -6 to -7°C .

Six experiments were made in which the "heat loss centre" was stimulated continuously for from $1\frac{1}{2}$ to 3 hours with the animals kept in the cold environment.

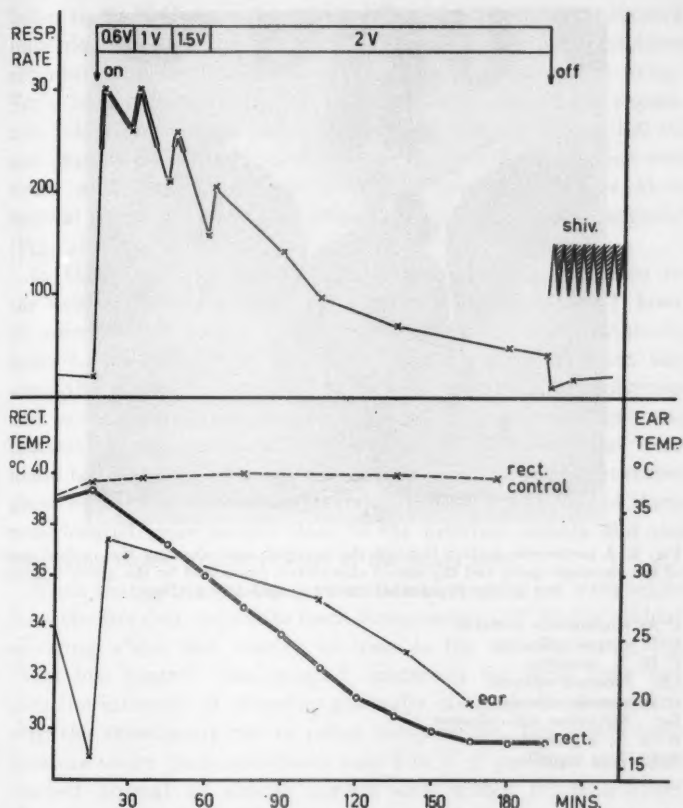


Fig. 1. Changes in respiration rate, rectal and ipsilateral ear surface temperature caused by three hours of unilateral electrical stimulation of the preoptic "heat loss centre" in an unanaesthetized goat kept in an environmental temperature of -6°C .

Rect. control: The rectal temperature of an intact animal kept for the same period of time in this environment.

Shiv.: The onset of violent shivering at the end of stimulation.

Double lining of respiration curve indicates duration of panting.

The most pronounced and persisting polypneic and vasodilatory effects were obtained in the experiment in which the stimulation in the freezing room was prolonged to last for 3 hours. The general course of this experiment is shown in Fig. 1. Although

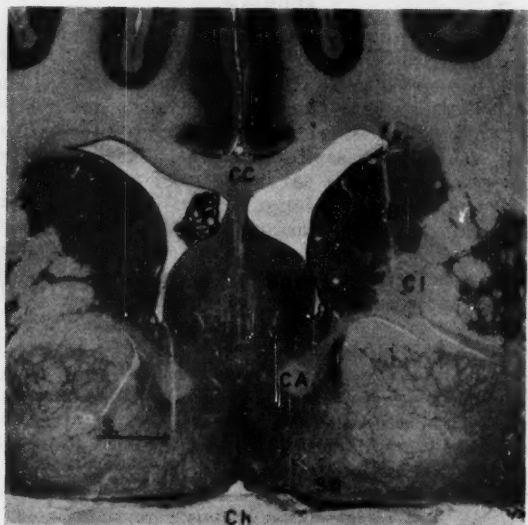


Fig. 2. A transverse section through the preoptic area showing the ventral part of the electrode track and the site of stimulation (indicated by the arrow labelled S.) in the experiment shown graphically in Fig. 1.

- C A: commissura anterior
- C C: corpus callosum
- C D: N. caudatus
- Ch: chiasma opticum
- C I: capsula interna
- fsc: fasciculus sub-callosus
- S O: N. supraopticus
- Spt: Area septalis

the initially very high respiratory rate gradually declined as the stimulation went on and the strength of stimulation had to be increased to maintain it high during the first half of the experiment, obvious polypnea was here observed during the entire period of stimulation. In spite of a 10°C drop of rectal temperature towards the end of the experiment, shivering was not observed as long as the stimulation lasted. Most violent shivering, however, did appear as soon as the period of stimulation was ended. Peripheral vasodilatation, as judged by ear surface temperature, persisted in the ipsilateral ear for more than two hours of stimulation. The vasodilatation in the contralateral ear was in this as in the other experiments of somewhat shorter duration. When towards the end of the experiment the rectal temperature had

fallen to 29.5° C the animal had difficulties in standing, showed muscular stiffness and attempts made to obtain blood samples revealed a markedly decreased blood flow in accessible veins. From having been 70 mg/100 ml at the beginning of the experiment the blood glucose had now reached a level of 200 mg/100 ml and glucose was present in the urine. The site of stimulation was below and slightly in front of the anterior commissure at a sagittal plane through the lateral border of the area septalis (Fig. 2).

In the other five experiments the periods of stimulation in the cold environment were of shorter duration (from 1 hour 15 minutes to 2 hours), causing a drop of rectal temperature of from 4.5 to 8° C. The stimulation was interrupted when the strength had to be increased to 3 V to maintain the polypnea and to suppress shivering, since stimulation of this strength was irritating to the animals. Here the points of stimulation were found to have been situated approximately at the same transverse plane as the site of stimulation shown in Fig. 2, but four of them were located more lateral close to the internal capsula and the remaining closer to the midline of the brain stem.

When the goats after the termination of stimulation were taken from the freezing room into room temperature (19° C) the violent shivering which had started as soon as the stimulation of the "heat loss centre" was stopped, continued for some time but then the intensity of shivering gradually diminished concomitant with the subsequent rise in rectal temperature. The rectal temperature under these conditions rose 3 to 4° C per hour and thus reached normal or almost normal level within 1½ to 3 hours after the goats were taken back into room temperature.

Discussion.

The experiments described here show that in spite of considerable lowering of the body temperature it is still possible to maintain heat loss mechanisms in action and to suppress the mechanisms concerned with the regulation against cold by electrical stimulation of the preoptic "heat loss centre". Thus in the most successful experiment it was possible to lower the rectal temperature of the goat 10° C by 2½ hours of stimulation and yet some polypnea persisted and shivering remained completely inhibited. It seems reasonable to suggest that in this animal the site of

stimulation (Fig. 2) had involved the activation of the largest number of structural elements concerned with the regulation against heat, which also might be expected from the fact that here the tip of the effective electrode was situated in the centre of that part of the preoptic area where ANDERSSON et al. (1956) found electrical stimulation to mobilize various heat loss mechanisms in the unanaesthetized goat.

Although in this experiment some polypnea persisted and shivering remained completely inhibited until the end of stimulation, the last half hour of stimulation did not cause any further drop of the rectal temperature below the 29.5°C it had reached after $2\frac{1}{2}$ hours of stimulation. The reason might be that as long as the goat remained conscious and in a standing position, her heat production and the insulation of the fur were towards the end of the experiment sufficient to balance the heat loss which now had become reduced due to the lowered body temperature and the reduced blood circulation in peripheral parts of the body.

Summary.

Polypnea, peripheral vasodilatation and inhibition of shivering were maintained for long periods by continuous electrical stimulation of the preoptic "heat loss centre" in unanaesthetized goats kept in an environmental temperature of -6 to -7°C . In this way the rectal temperature of one goat was lowered as far as to 29.5°C . This pronounced hypothermia was accompanied by drowsiness, hyperglycaemia and a reduced blood flow in accessible veins.

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The Development of the Mammary Gland in Normal and Castrated Male Rats after the Age of 21 Days.

By

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In most species the male mammary gland remains rudimentary throughout life and consists only of a restricted duct system at the base of the teat (FOLLEY 1952). However, a certain degree of mammary duct growth in the period between birth and puberty is reported for the male rat (MYERS 1917). The occurrence of further mammary development in the male rat at and/or after puberty may be inferred from the observations of TURNER and SCHULTZE (1931) who found a marked lobule proliferation in the mammary gland of the mature male. Development and secretion beginning at puberty in the male rat was also reported by McEUEEN, SELYE and COLLIP (1936). A more detailed description of the development of the mammary gland in male rats was given by ASTWOOD, GESCHICKTER and RAUSCH (1937). They found in the adult male rat mammary glands showing dense clusters of alveoli associated with a duct system with relatively little arborescence. They described this gland as "a small compact gland resembling in some way the gland in early pregnancy in the female, but differing in its restricted area, its irregular, imperfectly formed lobules and its thick walled incompletely developed acini".

Some information is also available about the age when this alveolar development begins. TURNER and SCHULTZE (1931) reported that lobule proliferation was absent in the males up to the age of 70—80 days. McEUEEN, SELYE and COLLIP (1936)

found considerable glandular development and secretion in a group of normal rats 48 days old, and ASTWOOD, GESCHICKTER and RAUSCH (1937) described the formation of alveoli in rats of 8 weeks.

Results obtained by studies of the effect of castration on the mammary development in male rats are somewhat conflicting. TURNER and SCHULTZE (1931) compared castrated male rats with normal males at monthly intervals and concluded that "castration neither inhibits nor hastens the characteristic lobule proliferation". McEUEEN, SELYE and COLLIP (1936) reported that castration prevented the proliferation of mammary glands in immature male rats and was followed by involution of the glands in adult males. In the investigation by ASTWOOD, GESCHICKTER and RAUSCH (1937) it was pointed out that it is necessary to make a sharp distinction between the growth of the duct system and the formation of alveoli when comparing the mammary glands from normal and castrated male rats. These authors found that the total area (= the extension of the duct tree) of the adult male gland was the same in normal and castrated rats, but that alveoli were absent in the glands from castrated rats. Thus the alveolar development seemed to be dependent on hormonal stimuli from the male gonads while the development of the ducts was independent of these stimuli. These results of ASTWOOD, GESCHICKTER and RAUSCH were later confirmed (SMITHCORS and LEONARD 1942, COWIE 1949). However, it was emphasized by COWIE and FOLLEY (1947) that occasionally alveoli may be found in mammary glands of adult male rats gonadectomized when sexually immature. These authors suggested that this might be due to an action of progesterone secreted by the adrenal cortex.

As indicated above, neither observations nor conclusions arrived at by different investigators studying the mammary development in normal or castrated male rats are in complete agreement with each other. This may be due to differences between the reaction of the mammary glands of different strains of rats. Another factor that might be involved is the difficulty to assess mammary gland growth accurately.

When a series of investigations concerning the effect of different hormonal stimuli on the male mammary gland was planned, it seemed therefore necessary to begin with a reinvestigation of the development of the mammary glands in normal and castrated male rats after the age of 21 days.

Experimental.

Male rats from a closed colony at our Institute were used. This strain of albino rats was inbred for a long time but because of a high offspring mortality, the strain was outbred 2 years ago by allowing the females to mate with males from a closed colony of piebald rats. The rats were fed *ad libitum* with a diet consisting of bread, dog biscuits, mixed grain, and fresh milk daily.

The mammary glands were studied at different ages from 21 to 115 days in 13 normal rats and 15 rats castrated at the age of 21–29 days. Experiments were done on littermates distributed between the two groups.

As far as possible the mammary glands were extirpated in the following order: the third right thoracic gland at the rat's age of 21–27 days, the third left thoracic gland at 53–60 days, the first right inguinal gland at 84–92 days and the left abdominal gland at 98–115 days.

In addition to these glands, the right abdominal gland was extirpated at a more advanced age from 3 of the 13 normal rats. From 2 other normal and 2 castrated older rats not included in the groups mentioned above, mammary glands were also examined.

The mammary glands were studied in whole mount preparations stained with galloxyanin chromalum (JACOBSON 1948). Instead of Canada balsam "Distrene" was used as mounting medium.

The whole mount preparations were studied under the microscope with magnifications of $35\times$ and $100\times$. When comparing and describing the kind and degree of glandular development, a semiquantitative method introduced by COWIE and FOLLEY (1947) and used in this laboratory since 1948 was used. However, the system of scoring was slightly modified in the present work and will therefore be given here.

Arborescence of Duct System.

- D — Restricted duct system showing little branching.
- 2D — Duct system of moderate extent showing intermediate degree of arborescence.
- 3D — Widespread duct system, showing high degree of arborescence.

Side Buds.

- B — Few present.
- 2B — Numerous side buds fairly evenly distributed.
- 3B — Ducts covered with side buds.

Club-Shaped End Buds Usually Deeply Staining.

- E — Few present.
- 2E — Numerous end buds irregularly disposed round the periphery.
- 3E — Very numerous end buds regularly disposed round the periphery.

Alveolar Development.

- A — Occasional alveoli present.
- 2A — Moderate alveolar development, evenly distributed.
- 3A — Ducts covered with alveoli.

Glands showing intermediate degrees of development were scored as, *i. e.*, 1—2 D and 2—3 D. Sometimes the side buds and the alveoli were numerous but unevenly distributed over the gland. When they were present only in the central part of the gland, they were scored as B_c and A_c, respectively, and when they were present only in the periphery they were scored as B_p and A_p, respectively. In some glands from castrated rats, the alveolar development was scored as "(1)" which is explained under "results". The basis for forming our estimates is illustrated by the photographs.

It is obvious that this semi-quantitative method, based on a subjective estimate of the degree of glandular development, cannot give as good informations about minor quantitative differences as the more objective techniques used for the rat by SILVER (1953) and for the mouse by FLUX (1954). However, the intention of this investigation was to study qualitative and large quantitative differences. Conclusions are drawn only from observations of changes which are obvious. A statistical evaluation of the results as performed by COWIE and FOLLEY (1947) was therefore omitted.

Besides the whole mount preparations some mammary glands from normal and castrated rats were studied in paraffin sections. The mammary glands to be sectioned were fixed in Bouin's solution. Parts of the glands were embedded in paraffin wax, cut into sections 10 μ thick and stained with hematoxylin eosin. The microscopic appearance of these sections is described in detail under "results" and illustrated in figs 15 to 22.

Results.

The observations made on the whole mount preparations of the mammary glands from the normal and castrated rats belonging to the different age groups are summarized in tables I and II. The results are illustrated by photographs in plates I to IV. The range of body weights corresponding to the different ages is also given.

Normal Rats.

Age 21—27 days (body wt 30—60 g). In 12 rats (table I, col. 2) the first mammary gland was examined at this age. This group of glands included 7 right and 3 left thoracic glands showing a restricted duct system (fig. 7). Two right abdominal glands had

a more developed duct system than the thoracic glands mentioned (fig. 1). As may be seen from the table a few side buds were present in 6 of the 12 glands examined and some end buds in two of them. No alveoli were found. No paraffin sections were studied.

Age 53—60 days (body wt 90—190 g). From all 13 rats (table I, col. 3) a second mammary gland was examined. This group included 10 left and 3 right thoracic glands showing a marked development compared with the thoracic glands extirpated at the foregoing age (figs 2 and 8). In 12 of the 13 glands the duct system was more extended, and in most glands there were also more side buds than at the younger age. Seven animals had developed end buds. In 2 of the glands alveoli were absent, but in the remaining ones the beginning of alveolar development was obvious. The first alveoli appeared to arise from large ducts in the central part of the glands.

The left thoracic gland in expt 2 covered a smaller area than the other thoracic glands included in this group (table I, col. 3 and fig. 8). However, this rat weighed only 90 g while most of the other rats weighed between 160 and 180 g at this age. As it is known that at least the infantile development of the mammary glands corresponds more to the weight than to the age of the animal (ASTWOOD *et al.* 1937) this reduced body weight can give the explanation of the small gland area in this rat.

In expt 12 the left inguinal gland was studied in paraffin sections. The various structures of this gland are described here in spite of the fact that only one gland was studied from this group. However, in both ducts and alveoli the main appearance of the individual cells was the same in this gland as in those examined at the following ages. The epithelium lining the inner wall of the ducts was simple, composed of large cuboidal or low columnar cells with an abundance of cytoplasm. The nuclei of these cells, usually lying somewhat toward the basis of the cells, were round or ovoid. In some of the cells the cytoplasm contained vacuoles and in these cells the basal localisation of the nuclei was more marked. The nuclei contained one or more prominent nucleoli and showed a loose network of chromatin. Some groups of alveoli were present (fig. 15). The epithelium lining the alveoli was similar to that found in the ducts, but most of the cells in the alveoli had large vacuoles in the cytoplasm. These cells, because of the vacuoles, appeared swollen and in

many of them the apical ends tended to bulge into the lumen, which in most alveoli was very narrow. Some of the ducts and a few alveoli were slightly distended with darkly stained secretion.

Age 84—92 days (body wt 190—280 g). From 11 rats (table I, col. 4) a third mammary gland was examined. This group comprised 8 inguinal and 3 abdominal glands. All the glands showed a moderately extended duct system with an intermediate degree of arborescence. As may be seen from the table a few side buds were present in all the glands but the number of them seemed to be the same or less than in the glands removed previously. Only 3 of the 11 glands showed end buds. Alveolar development was more marked. One of the 11 glands had only a few alveoli, but the other glands presented thick ducts which began to be covered with alveoli (fig. 3).

Paraffin sections of a mammary gland were studied in expts 4, 11 and 13 (left inguinal gland) and in expt 8 (right abdominal gland). Compared with the observations at the preceding age the most striking change was an increase in size and number of the alveoli. Some ducts were seen which were surrounded by groups of alveoli (fig. 16). The appearance of the individual cells was similar to that described above but in some ducts and alveoli the epithelium was composed of two or several layers of cells. In a number of alveoli lumina appeared to be absent. Most of the cells had large vacuoles in the cytoplasm. A slight secretion was seen in some of the ducts and alveoli.

Age 98—115 days (body wt 215—295 g). In 11 rats (table I, col. 5) a fourth mammary gland was examined. This group included 9 abdominal and 2 inguinal glands. The duct system of these glands covered about the same area and showed a similar arborescence as those examined at the foregoing age. Most of the ducts were covered with dense clusters of alveoli (fig. 9). The restricted area, the thick ducts, and the numerous clusters of alveoli made the appearance of the glands very compact. In the periphery of some glands the alveolar development was less extensive and the ducts showed a few side buds. The left abdominal gland in expt 9 was unequally developed and, therefore, no scoring was made in the table. The largest part of this gland had thick ducts covered with alveoli, but in part of the periphery the alveolar development was scarce and both side buds and end buds were present. This gland was the only one presenting end buds.

Sections were studied in expts 1, 2, 3 and 8 (left inguinal gland) and in expts 6 and 7 (right abdominal gland). In all the sections a large number of alveoli were seen. As in the foregoing age group the epithelium in most of the ducts and alveoli was composed of two or more layers of cells. In fact, some parts of the glands presented such an abundant proliferation of the epithelial cells within ducts and alveoli that they appeared more as solid cords and clumps of large cells than as ducts and alveoli (fig. 17). The individual cells were similar to those described for the group of 53—60 days old rats.

In addition to the mammary glands in the four age groups mentioned above and summarized in table I, some other glands were examined in older normal rats. The right abdominal gland was examined in expts 9 and 10 (table I) at the rat's age of 174 days and in expt 1 (table I) at the age of 253 days. In two other normal rats not included in the groups mentioned above right thoracic glands were examined at the age of 279 and 390 days, respectively. All the five glands from these older rats covered about the same area and showed about the same number of alveoli as those examined at the age of 98—115 days. But since some of the ducts and most of the alveoli seemed to be slightly dilated, the glands appeared less compact (fig. 13). However, some of the alveoli and most of the ducts had the same appearance as at the age of 98—115 days and true cyst formations were not found.

The paraffin sections of the mammary glands from these older rats revealed a number of ducts and alveoli distended with darkly stained secretion (fig. 18). In all the ducts and most of the alveoli the epithelium was composed of a single layer of cells. Solid cords and clumps of epithelial cells, dominating the picture at the age of 98—115 days, were only rarely found. A marked reduction in the height of the epithelium to a low cuboidal form was observed in the most distended ducts and alveoli. In these cells no vacuoles were seen in the cytoplasm. It should, however, be pointed out that the majority of the alveolar cells were still large and cuboidal with vacuoles in the cytoplasm.

Castrated Rats.

Age 21—27 days (body wt 35—55 g). In 11 rats (table II, col. 3) the first mammary gland was examined at this age. This group of glands was composed of 9 right and 2 left thoracic

Table I.
The development of the mammary glands in normal, male rats.

	Right thoracic gland*				Left thoracic gland*				Right inguinal gland*				Left abdominal gland*										
	Age at exam. days		Development**		Age at exam. days		Development**		Age at exam. days		Development**		Age at exam. days		Development**								
	D	B	E	A	D	B	E	A	D	B	E	A	D	B	E	A							
1. (a)**	26	1	0	0	0	54	1-2	2	2	0	2	2	85	2	1-2	0	2	2	1	0	2-3		
2. (b)	27	1	0	0	0	55	1	1	2	0	0	0	86	2	1	1	1	1	2	1	0	2-3	
3. (c)	26	1	1	0	0	54	1-2	1	0	1-2	2-3	3	85	1-2	1	0	2-3	2	2	0	0	3	
4. (d)	24	1	0	0	0	54	1-2	1	1	1-2	2 ^c	—	85	2	1	1-2	2 ^c	—	—	—	—	—	
5. (d)	24	1	0	0	0	54	1-2	2	0	1-2	2	—	85	2	1-2	0	2	—	—	—	—	—	
6. (e)	—	—	—	—	—	53	1-2	1	1	1	1	—	84	1-2	1	0	2	106	2	0	0	3	
7. (e)	22	1	0	0	0	53	1-2	2	2	1	1	—	—	—	—	—	—	108	2	1	0	2-3	
8. (f)	127	1	1	0	0	*60	1-2	1	0	1	1	—	89	2	1	0	1-2	107	2	0	0	3	
9. (f)	127	1	1	0	0	*60	1-2	1	0	1-2	2-3 ^c	—	89	2	1-2 ^p	1	2-3 ^c	107	see "results"				—
10. (f)	127	1	1	1	0	*60	1-2	1	0	1	1	—	—	—	—	—	—	107	2	1	0	2-3	
11. (g)	*26	1-2	1	0	0	54	1-2	1	0	1-2	—	—	*87	2	1	0	2-3	*107	2	1	0	2-3	
12. (g)	21	1	0	0	0	54	1-2	1	1	1	1	—	*87	2	1	0	2-3	*108	2	1	0	2-3	
13. (g)	*26	1-2	1	1	0	54	2	1-2	0	1	1	—	*87	2	1	0	2-3	*108	1-2	1	0	2-3	

* exceptions are indicated as follows:

1 = left thoracic gland.

2 = right thoracic gland.

3 = left abdominal gland.

4 = right abdominal gland.

5 = right inguinal gland.

** abbreviations see "experimental".

Table II.
The development of the mammary glands in castrated, male rats.

Expt	Age at castr., days	Right thoracic gland*				Left thoracic gland*				Right inguinal gland*				Left abdominal gland*			
		Age at exam. days	Development**			Age at exam. days	Development**			Age at exam. days	Development**			Age at exam. days	Development**		
			D	B	E A		D	B	E A		D	B	E A		D	B	E A
1. (a)**	28	26	1	0	0 0	54	1-2	1	2 0	85	2	1	0 (1)	98	2	1	0 1
2. (b)	29	27	1	0	0 0	55	1	1 2 (1)		86	2	1	0 (1)	99	2	1	0 1
3. (b)	29	27	1	0	0 0	55	1-2	1 1-2 0		—	—	—	—	—	—	—	—
4. (c)	28	26	1	0	0 0	54	1-2	1 2 0		—	—	—	—	—	—	—	—
5. (d)	25	25	1	0	0 0	55	1-2	1 1-2 0		86	2	1	0 (1)	—	—	—	—
6. (d)	25	25	1	0	0 0	55	1-2	1 0 (1)		—	—	—	—	—	—	—	—
7. (e)	22	22	1	0	0 0	53	1-2	1 2-3 0		84	2	1	0 (1)	105	2	1	0 (1)
8. (e)	22	22	1	0	0 0	53	1-2	1 2 0		84	2	1	0 0	105	2	1	0 (1)
9. (f)	27	27	1	1	0 0	56	2	1 0 1		89	2	1	0 1	107	2	1	0 1
10. (f)	27	27	1	1	0 0	56	1-2	1 0 (1)		89	1-2	1 0 (1)		—	—	—	—
11. (g)	21	21	1	0	0 0	54	1-2	1 0 (1)		*87	2	1	0 (1)	*107	2	1	0 (1)
12. (g)	21	21	—	—	—	54	1-2	1 1-2 0		*87	1-2	1 0 (1)		*107	1-2	1 0 (1)	
13. (h)	21	21	—	—	—	—	—	—		*92	2	1	0 1	*115	2	1	0 1
14. (h)	21	21	—	—	—	—	—	—		*92	1-2	1 0 1		115	2	1	0 1
15. (h)	21	21	—	—	—	—	—	—		*92	1-2	1 0 (1)		*115	2	1	0 (1)

* exceptions are indicated as follows:

- 1 = left thoracic gland.
2 = right thoracic gland.
3 = left abdominal gland.
4 = right abdominal gland.
5 = right inguinal gland.

** abbreviations see "experimental".

glands. As may be seen from the table these glands were extirpated 2 days before or at the same day as the rats were castrated. Thus, these glands are extirpated from "normal" rats and may also be included in the corresponding age group of the normal rats mentioned above. All the glands had a restricted duct system (figs 4 and 10) and 2 of them showed a few side buds. End buds or alveoli were not found. No paraffin sections were studied.

Age 53—60 days (body wt 120—165 g). From 12 rats a second gland was examined (table II, col. 4). This group included 10 left and 2 right thoracic glands showing a marked development compared with those examined at the foregoing age (figs 5 and 11). All the glands had a few side buds and most of them presented end buds. In fact, at this age the development of the duct system, side buds and end buds seemed to be the same in the castrated as in the normal rats. Alveoli were not found in 7 of the 12 glands while in the remaining glands occasional alveoli were present. Contrary to the mode of alveolar development in the normal rats, most of these alveoli developed at the end of the smallest branches of the duct system and the ducts were not thickened. In table II the scoring "(1)" was used to describe this distribution of very few alveoli in combination with normal (= not thickened) ducts.

In expt 2 the left thoracic gland covered a smaller area than the other glands included in this group (table II, col. 4, and fig. 11). As this rat weighed 120 g and most of the other rats weighed between 145 and 165 g at this age, the explanation of this restricted gland area may be the same as that discussed for one gland in the corresponding age group of the normal rats.

From none of these 12 rats microscopical examinations of paraffin sections were made. But from 3 other rats, castrated at the age of 21—28 days and not included in this group, sections of the right thoracic gland were studied when the rats were 68 days old. The dominating structures in these glands were ducts of different sizes (fig. 19). All the ducts had a simple epithelium composed of small cuboidal cells without vacuoles in the larger ducts and of larger cuboidal cells, a number of which had vacuoles in the cytoplasm, in the smallest ducts. A few small groups of alveoli were also present (fig. 21). The epithelium lining these rare and small alveoli was simple and composed of large cuboidal cells with vacuoles in the cytoplasm. The nuclei of the cells in both ducts and alveoli were similar to those described

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for the normal rats. Secretion was very slight, and the lumina of ducts and alveoli were narrow.

Age 84—92 days (body wt 190—260 g). From 12 rats (table II, col. 5) a third gland was examined. This group comprised 7 right inguinal, 2 left abdominal and 3 left thoracic glands showing a duct system of moderate extent and intermediate arborescence (fig. 6). All the glands had a few side buds, but end buds were not found. A few alveoli were present in 11 of the 12 glands. As in the glands of the previous age group most of these alveoli arose from the smallest branches of the ducts. However, in expts 9, 13 and 14 (table II, col. 5) a few alveoli appeared around large ducts in the central part of the glands (fig. 14). A comparison of the glands of this age group with the glands of the corresponding age group of the normal rats showed that the development of the duct system was about the same but that the alveolar development was extensive in the normal rats and only slight in the castrated rats.

Paraffin sections were studied from the left inguinal gland in expt 5 (table II, col. 5) and from the left abdominal gland in expt 13 (table II, col. 5). These sections showed essentially the same as those of the foregoing age group. As expected from the whole mount preparations the mammary gland in expt 13 presented several groups of alveoli (fig. 20). The epithelium lining these alveoli was simple and composed of large cuboidal cells with vacuoles in the cytoplasm (fig. 22).

Age 98—115 days (body wt 215—280 g). From 10 rats (table II, col. 6) a fourth mammary gland was examined. This group was composed of 6 left abdominal, 3 right abdominal and 1 right inguinal gland, which showed the same type and degree of development as those extirpated at the foregoing age. All the glands presented a few alveoli, but when compared with those examined at the corresponding age from the normal rats, this alveolar development appeared very limited.

From 7 of the 10 rats paraffin sections of abdominal or inguinal glands were studied. The appearance of the ducts and the few alveoli was similar to that described for the glands examined at the foregoing age.

In addition to the mammary glands summarized in table II and described above, some glands from two other castrated rats were studied. From these rats, castrated at the age of 21 days, whole mount preparations of the right abdominal glands and

paraffin sections of the left abdominal glands were studied at the rats' age of 202 days. These glands presented the same development and picture as those extirpated from the other castrated rats at the age of 98—115 days.

Comments.

Development of the duct system. In the present work the extension of the duct system was estimated only by studying the whole mount preparations under the microscope. Objective measurements of the gland area or the degree of branching of the ducts were not performed. With our method it was not possible to reveal eventual differences in the development of the duct system between the glands from the normal and castrated rats. ASTWOOD et al. (1937) using a similar method also found that the area of the mammary glands from adult intact rats was the same as in castrated male rats. COWIE (1949) measured the total gland area per rat up to the age of 100 days and even with this method it was not possible to detect any significant difference between the mammary duct system from intact males and males castrated at the age of 22 days.

A comparison between the development of the mammary duct system in the same animal at different ages is possible when corresponding glands are studied. In this work this is done only in the first two age groups (21—27 days and 53—60 days, respectively). All the third thoracic glands included in the first age group (fig. 4) showed a few very short ducts with a few or no side buds; end buds were only present in 2 of the 23 glands. Most of the corresponding glands included in the second age group (fig. 5) presented a much larger area, side buds and end buds. Thus, a marked development of the duct system took place both in the normal and castrated rats between the age of 21 and 60 days. The other age groups are mostly composed of abdominal and inguinal glands, which are not quantitatively comparable to the thoracic glands. A further development of the duct tree after the age of 60 days can therefore not be evaluated from this work. ASTWOOD et al. (1937) reported that the growth of the ducts ceased after the age of 8 weeks in both normal and castrated male rats, but this report is not in agreement with the work of COWIE (1949) who related the mammary gland area to that of the body surface in normal and castrated male rats up to the

age of 100 days. COWIE found that the growth of the duct tree continued during this period of life.

Differences in the appearance of the individual mammary ducts were found between normal and castrated rats. At about 3 months the mammary ducts in most of the normal rats seemed thick in whole mount preparations (figs 3 and 9), and paraffin sections revealed that the epithelium lining the inner wall of the ducts was composed of 2 or several layers of large epithelial cells (fig. 17). In the glands from castrated rats the epithelium in the ducts was simple.

In 5 mammary glands extirpated from normal rats at a more advanced age (174—390 days), some of the ducts were dilated and distended with secretion (fig. 18). In 2 glands from castrated rats 202 days old, no such dilatation of the ducts was seen.

Development of the alveoli. In agreement with the investigations of ASTWOOD et al. (1937) and COWIE (1949), a difference was found between the alveolar development of the mammary glands in normal and castrated male rats. Already by the age of 53—60 days most of the normal rats presented glands with alveoli along the ducts. At the age of 98—115 days all the normal rats had mammary ducts covered with dense clusters of alveoli (figs 9 and 17). Castrated rats occasionally had a few small and isolated lobules of alveoli at 53—60 days and in older animals all glands showed a few alveoli (figs 14, 20 and 22). However, after castration the alveolar development was very limited compared with that occurring in normal rats.

A difference between mammary glands from normal and castrated rats was also found as regards the site and type of alveolar formation. In normal animals clusters of alveoli developed close to each other along the sides of the ducts, but in castrated animals the few and isolated groups of alveoli developed at the end of the smallest branches of the ducts. In the normal rats of about 3 months the epithelium in most of the alveoli was composed of several layers of large cells, and in many glands the alveoli appeared as solid clumps of cells (fig. 17), but in all the glands from the castrated rats the epithelium of the alveoli was simple (figs 20 and 22).

In the 5 older, normal rats some of the alveoli were slightly distended with secretion. The glands from the 2 older castrated rats had only a few, non-dilated alveoli.

Discussion.

The main features shown by the mammary glands of normal adult male rats studied in the present work are the same as those described by ASTWOOD et al. (1937). The glands had 1) a small area, 2) an extensive alveolar development, and 3) several layers of epithelial cells lining ducts and alveoli. The most striking feature was the thickness of the epithelium in the ducts and alveoli. Such a proliferation of epithelial cells in the mammary gland has to our knowledge not been observed in female or castrated male rats. That injections of testosterone into intact or castrated female and castrated male rats can stimulate a development of mammary gland structures characteristic for normal adult male glands has been described by *e. g.* ASTWOOD et al. (1937) and confirmed for castrated rats by AHRÉN and ETIENNE (1958). Thus, in the rat hormones of the male gonads or injections of testosterone can promote both a development of alveoli and a proliferation of the epithelial cells lining the ducts and alveoli. The epithelium of the monkey mammary gland stimulated by androgenic hormones seems to show a similar reaction. FOLLEY, GUTHKELCH and ZUCKERMAN (1939) observed in the testosterone treated male monkey that the duct epithelium in many places was heaped and showed papillomatous growth into the lumina. VAN WAGENEN and FOLLEY (1939) found the same picture in castrated female monkeys injected with testosterone. However, to our knowledge, a similar effect of androgenic hormones on the epithelial cells of the mammary gland has not been described for other species.

As commented upon above, the extension of the duct system of the mammary glands seemed to be the same in normal and castrated rats. The ducts in the normal rats were covered with dense clusters of alveoli. In the younger castrated rats alveoli were found occasionally. However, in all the glands examined from castrated rats older than 3 months a few groups of small alveoli were found. These alveoli did not appear along the main ducts as in the normal rats, but at the end of the smallest branches of the duct system. The epithelium lining these alveoli was simple. In fact, these alveoli were more similar to those developing in normal female rats or promoted by ovarian hormones than to those developing in normal male rats or promoted by testosterone.

The last mentioned observations confirm and extend those reported by COWIE and FOLLEY (1947) and COWIE (1949).

Summary.

The development of the mammary glands was studied in normal male rats and male rats castrated at the age of 21—29 days. The main results were as follows:

1) At the age of 21—27 days the glands showed only a restricted duct system.

2) At the age of 53—60 days the development of the ducts, side buds and end buds was the same in normal and castrated rats. In some of the glands from normal rats alveolar development was obvious. In the castrated rats small groups of alveoli were found occasionally only.

3) At the ages of 84—92 days and 98—115 days the mammary gland area seemed to be the same in normal and castrated rats. The alveolar development was extensive in the normal rats and only scarce in the castrated rats.

4) The epithelium lining ducts and alveoli of the glands was simple in the younger normal and in all the castrated rats but composed of two or more layers of cells in most of the normal rats at the age of about 3 months.

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Comments to Plates I-IV.

The figures illustrate observations made on mammary glands from normal and castrated male rats. The whole mount preparations also show the basis for forming our estimates of the glandular development (for the abbreviations see "experimental"). Figs 1-14 are photographs of whole mount preparations taken with the same magnification (see plate IV). Figs 15-22 are microphotographs of 10 μ thick sections through glands stained with hematoxylin eosin. Figs 15-20 and figs 21-22 are taken with the same magnifications, respectively (see plate IV).

Plate I.

Fig. 1. Normal rat (expt 13, table I), 26 days old. Left abdominal gland, scored as 1-2D 1B 1E.

Fig. 2. Same rat as in fig. 1, 54 days old. Left thoracic gland, scored as 2D 1-2B 1A.

Fig. 3. Same rat as in figs 1 and 2, 87 days old. Left abdominal gland, scored as 2D 1B 2-3A.

Fig. 4. Castrated rat (expt 11, table II), 21 days old. Right thoracic gland, scored as 1D.

Fig. 5. Same rat as in fig. 4, 54 days old. Left thoracic gland, scored as 1-2D 1B (1)A.

Fig. 6. Same rat as in figs 4 and 5, 87 days old. Left abdominal gland, scored as 2 D 1B (1)A.

Plate II.

Fig. 7. Normal rat (expt 2, table I), 27 days old. Right thoracic gland, scored as 1D.

Fig. 8. Same rat as in fig. 7, 55 days old. Left thoracic gland, scored as 1D 1B 2E.

Fig. 9. Same rat as in figs 7 and 8, 99 days old. Left abdominal gland, scored as 2D 1B 2-3A.

Fig. 10. Castrated rat (expt 2, table II), 27 days old. Right thoracic gland, scored as 1D.

Fig. 11. Same rat as in fig. 10, 55 days old. Left thoracic gland, scored as 1D 1B 2E (1) A.

Fig. 12. Same rat as in figs 10 and 11, 99 days old. Left abdominal gland, scored as 2D 1B 1A.

Fig. 13. Normal rat (not included in the tables but discussed under "results"), 390 days old. Right thoracic gland showing a duct system of moderate extent and ducts covered with clusters of alveoli. Note that this gland appears less dense than the glands shown in figs 3 and 9.

Fig. 14. Castrated rat (expt 13, table II), 92 days old. Left thoracic gland, scored as 2D 1B 1A.

Plate III.

Fig. 15. Normal rat (expt 12, table I), 54 days old. Left inguinal gland showing a duct and a group of alveoli with simple epithelium. Note the vacuoles in the cytoplasm of the alveolar cells.

Fig. 16. Normal rat (expt 13, table I), 87 days old. Left inguinal gland showing a large duct surrounded by alveoli. Epithelial cells with large vacuoles in the cytoplasm. Slight secretion.

Fig. 17. Normal rat (expt 6, table I), 106 days old. Right abdominal gland showing part of a large duct lined by several layers of epithelial cells, and groups of alveoli with abundant proliferation of the epithelium. Many of the alveoli appear to lack lumina.

Fig. 18. Normal rat (not included in the tables but the same as in fig. 13), 390 days old. Left thoracic gland showing ducts and alveoli lined by a simple epithelium and distended with secretion.

Plate IV.

Fig. 19. Castrated rat (not included in the tables but discussed under "results"), 68 days old. Right inguinal gland showing ducts lined by a single layer of small epithelial cells.

Fig. 20. Castrated rat (expt 13, table II), 92 days old. Left abdominal gland showing some ducts and small groups of alveoli.

Fig. 21. Same gland as in fig. 19. A small duct and a small group of alveoli. A few vacuoles in the epithelial cells of the alveoli.

Fig. 22. Same gland as in fig. 20. A group of small alveoli lined by a single layer of epithelial cells. In some of the cells vacuoles in the cytoplasm.

Plate I.



Fig. 1.



Fig. 4.

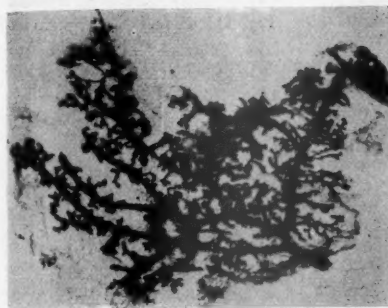


Fig. 2.

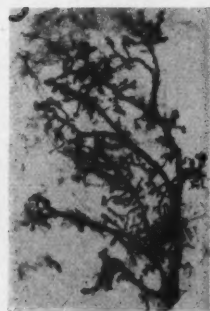


Fig. 5.



Fig. 3.



Fig. 6.

AHRÉN AND MONIQUE ETIENNE: Development of the Mammary Gland.
Acta physiol. scand. 41: 2—3.

Plate II.



Fig. 7.



Fig. 8.



Fig. 11.



Fig. 10.



Fig. 9.

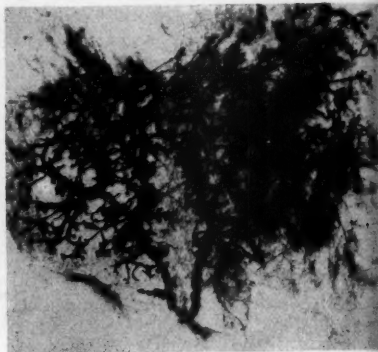


Fig. 12.

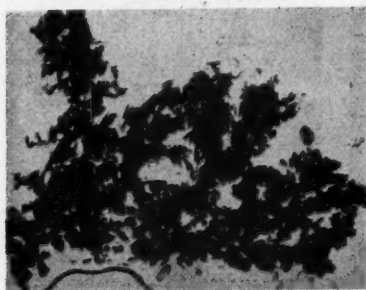


Fig. 13.



Fig 14.

Plate III.



Fig. 10.



Fig. 15.



Fig. 17.



Fig. 16.

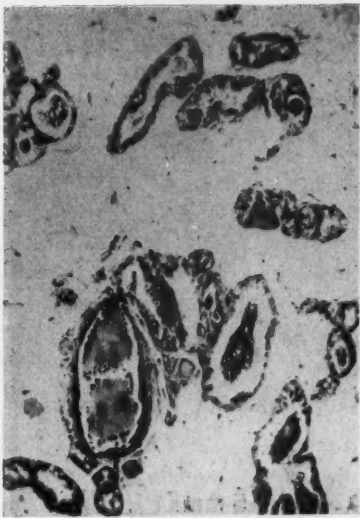


Fig. 18.

AHRÉN AND MONIQUE ETIENNE: Development of the Mammary Gland.
Acta physiol. scand. 41: 2—3.

Plate IV.

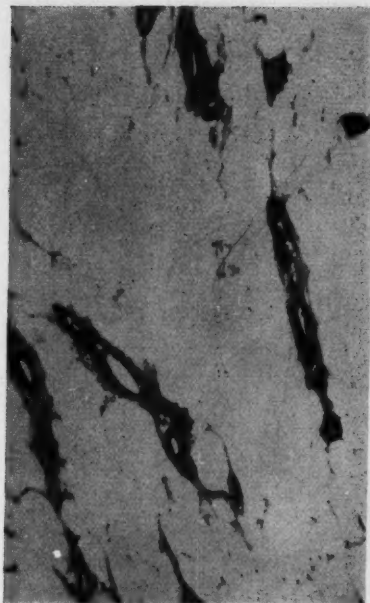


Fig. 19.



Fig. 20.

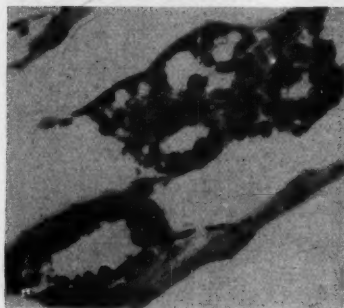


Fig. 21.

5 mm.
—
(whole mounts)

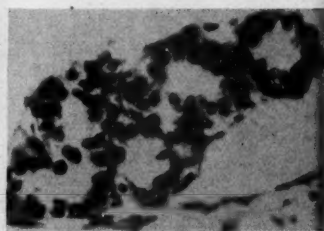


Fig. 22.

0.40 mm.
—
(microph. Figs. 15-20)

0.10 mm.
—
(microph. Figs. 21-22)

From the Department of Physiology, Veterinary College of Norway,
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Species Differences of Clotting Factors in Ox, Dog, Horse, and Man.

Thromboplastin and Proconvertin.

By

HELGE STORMORKEN.

Received 6 August 1957.

That the effect of thromboplastin depends on species origin has been known since the earliest period of coagulation research (see the reviews by WÖHLISCH 1929 and by MORE, SUNTZEFF and LOEB 1935). However, most of the work on this problem was done before the concept of two systems of the clotting of blood arose, the extrinsic and the intrinsic system. The earlier work is therefore often difficult to interpret in the light of present day knowledge.

Thromboplastin forms a complex, *i. e.* convertin, with calcium and proconvertin (see references by DEUTSCH 1955 and HJOET 1957). With this in mind the specificity of thromboplastin could then be attributed to a definite reaction. The problem has since been studied by BURSTEIN (1950), and MANN and HURN (1952).

Although the specificity of thromboplastin has been known, its influence on the interpretation of the results of clotting studies has often been overlooked. It probably explains some of the discrepancies between findings from different laboratories since preparations of different species origin are commonly used, for instance bovine lung, rabbit brain and lung, and human brain.

The purpose of the present work was to study the influence of the species specificity on the thromboplastin-proconvertin reaction, and to explore to what extent the available methods for proconvertin assay may be used in different species.

Materials and Methods.

a) Materials.

<i>Saline brain thromboplastin</i>	} Prepared as described by HJORT (1957).
<i>Prothrombin</i>	
<i>Proconvertin</i>	
<i>Bovine fibrinogen</i>	
<i>Dilution fluid I and II</i>	

Proconvertin-deficient citrated human plasma was obtained from a patient with congenital defect of this factor, but with a normal content of other clotting factors (OWREN 1952).

Proaccelerin-deficient citrated plasma was prepared from a patient with parahemophilia (OWREN 1947).

Buffer. The modified veronal buffer of OWREN (1947) was used. pH 7.35, ionic strength 0.154.

Phenylindanedione. (PID). The preparation "Thrombantin", Nyegaard & Co, Oslo, was used. A 3 per cent solution for intravenous administration was kindly prepared by the same factory.

Anticoagulants. Sodium citrate dihydrate, 3.13 per cent (w/v), or potassium oxalate monohydrate 2.5 per cent (w/v) was used.

Barium sulphate (C. p. "Baker").

Speedex (amorphous diatomaceous SiO_2) Dicalite Division, Great Lakes Carbon Corporation, New York.

Asbestos filters were obtained through Gallus & Co., Oslo, Norway.

Silicon. The glassware were coated with General Electric Dri-Film 9987 according to the method of TOCANTINS (1955). Two coatings were used. The needles were coated with Arquad 2-C (Armour Co., Chicago) by boiling for two minutes in a 2 per cent solution.

Heparin. A preparation manufactured by A/S Apotekernes Laboratorium, Oslo, containing 5,000 units per ml was used.

b) Methods.

Prothrombin assay. a) The Russell's viper venom-cephalin method was used as described by HJORT, RAPAPORT and OWREN (1955), except that the substrate plasma was dialyzed against saline to remove the oxalate.

b) Three-stage prothrombin method (STORMORKEN 1957).

Proconvertin was assayed essentially according to AAS (1952), using human proconvertin-deficient plasma as the substrate plasma. To avoid influence from proaccelerin-acceleriner in testing different species, 0.1 ml of a bovine acceleriner preparation was added to the system. This preparation contained about ten times the normal human plasma activity. The system was as follows: 0.2 ml thromboplastin + 0.2 ml proconvertin-deficient plasma + 0.1 ml acceleriner + 0.2 ml diluted test plasma + 0.2 ml calcium chloride. The thromboplastin used was homologous to the test plasma.

Converitin. The same method as for proconveritin was used, except that the thromboplastin was omitted. The correlation graph was steeper than that for proconveritin.

P & P method. The method for assay of the combined activity of proconveritin and prothrombin activity was used as described by OWREN and AAS (1951), except that the substrate plasma was the same as that for the Russell's viper venom test. Test system: 0.2 ml thromboplastin + 0.2 ml adsorbed, dialyzed, bovine plasma + 0.2 ml test plasma (1/10 dilution) + 0.2 ml calcium chloride, optimal concentration.

Collection of blood. Blood from the different species was obtained by venepuncture, and the blood was allowed to flow freely into the tube or bottle containing the anticoagulant. The first few ml were always discarded, and "silicone" blood was refrigerated throughout.

Centrifugation. The centrifugations were performed with an International Refrigerated Centrifuge, model PR-2 (International Equipment Co., Boston). At 2,500 rpm the maximal g was 1,400.

Glass activation. The plasma was activated with Speedex, one fourth of the plasma volume, for 5 minutes at room temperature under continuous tilting. The powder was removed by centrifugation at 2,500 rpm for 30 minutes.

Adsorption. Oxalated plasma was adsorbed for 10 minutes at room temperature with 100 mg per ml plasma of barium sulphate under continuous stirring, and the adsorbent removed by centrifugation at 2,500 rpm for 30 minutes.

Storage. Reagents stored for more than one day were kept frozen at -20°C .

Experiments.

1. The Species Specificity of Thromboplastin.

No special test system is needed to demonstrate the specificity of thromboplastin. It is clearly shown with the thromboplastin time, and even better with the P & P method of OWREN and AAS (1951), due to the dilution of the test plasma. Tables 1 and 2 show the results of an experiment with plasmas and thromboplastins from four different species using these two methods. The plasmas were drawn with careful silicone technique and the thromboplastins were prepared with one and the same method. The extracts were diluted with veronal buffer to optimal effect on the homologous plasma.

The tables show marked differences in the activity of the various thromboplastins. With one exception the homologous combinations gave the shortest clotting times. The exception was found with dog plasma, where human thromboplastin was slightly more active than the homologous. This difference was constant with

Table 1.
*The thromboplastin times of different plasmas
with different thromboplastins.*

Thromboplastin species	Plasma species			
	Human	Ox	Horse	Dog
Human	14.0	22.8	14.2	9.0
Ox	33.1	13.5	17.5	10.8
Horse	25.8	43.8	10.1	11.0
Dog	32.2	20.0	19.0	10.4

Clotting mixture: 0.2 ml thromboplastin + 0.2 ml plasma + 0.2 ml CaCl_2 , optimal concentration. Clotting times in seconds.

Table 2.
*Clotting times of different plasmas in the P & P
method with different thromboplastins.*

Thromboplastin species	Plasma species			
	Human	Ox	Horse	Dog
Human	31.5	132	33.1	24.0
Ox	66.2	40.0	35.5	25.5
Horse	85.2	162	25.8	39.5
Dog	65.5	65.0	64.0	25.0

Clotting times in seconds.

three different batches of thromboplastin. — One might therefore expect that dog thromboplastin would be as active as human thromboplastin in human plasma, but the tables show that it was not. This finding may suggest that another factor with a different pattern of specificity takes part in the thromboplastin-proconvertin reaction.

2. Attempts to Find a Suitable Substrate Plasma for the Assay of Proconvertin in Different Species.

Different methods for the assay of proconvertin exist, and they are more or less specific for proconvertin. The best one probably is the method of Aas (1952) which uses plasma from a patient with congenital deficiency of proconvertin as the substrate plasma. Even this method is only specific for proconvertin in the lower range of clotting times, because the intrinsic system in-

Table 3.

The thromboplastin time of different thromboplastins in the test for proconvertin activity using proconvertin-deficient human plasma.

Thromboplastin species	Human	Ox	Horse	Dog
Clotting time, seconds	64.5	74.0	58.5	82.6

Clotting system: 0.2 ml proconvertin-deficient plasma + 0.2 ml thromboplastin + 0.1 ml accelerin + 0.2 ml dilution fluid II + 0.2 ml CaCl_2 , 30 mM.

creasingly influences the results as the clotting times approach the buffer value. The buffer value (time), *i. e.* the thromboplastin time of the proconvertin-deficient plasma, might be regarded as the partial thromboplastin (cephalin-)time of the plasma, and consequently is the result of an activity derived from the intrinsic system. This implies that the dilution curve results from activity of both systems. In experiments with different thromboplastins the buffer time was found to vary with the thromboplastin species, indicating that the cephalin activity of the thromboplastins also shows species specificity. This was verified in experiments with cephalin prepared from different species (unpublished data). Long buffer times are, of course, advantageous because of the decreased influence of the intrinsic system. In table 3 are shown the buffer times with different thromboplastins in the proconvertin assay system used in this study. Although this method must be recommended as the most accurate one, its usefulness is restricted, because the proconvertin-deficient plasma is not easily available. The following experiments were therefore performed to prepare a more generally available substrate plasma for the assay of proconvertin. With the results given in tables 1 and 2 in mind, homologous thromboplastins were used in these experiments.

A. *Experiments with phenylindanedione.* Several reports have dealt with the dissociation of prothrombin and proconvertin in the early stages of treatment with dicumarol-like drugs (see DEUTSCH 1955, p. 100). Proconvertin is reduced more quickly than is prothrombin, and this finding has been used to prepare a substrate plasma for proconvertin assay, for instance by MANN and HURN (1951), and BIGGS and MACFARLANE (1953, p. 82).

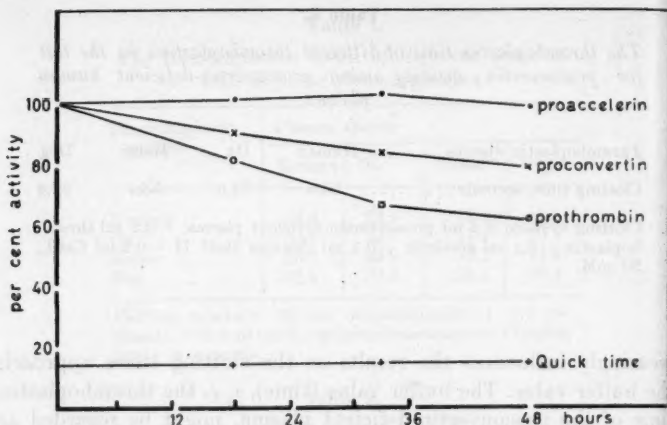


Fig. 1. The effect on the prothrombin and proconvertin level in plasma from a cow given phenylindanedione intravenously. Each column at the abscissa indicates the injection of 6 g phenylindanedione intravenously.

Since the dissociation probably is due to a shorter survival time of proconvertin than of prothrombin, large doses of the drug should be given to make the gap between the two factors as large as possible by a total block of the synthesis.

It was thought that the cow would serve this purpose excellently with its low activity of proconvertin as judged both from the P & P method and the proconvertin assay using homologous thromboplastin. A cow was fed 10 g of PID perorally every 6th hour for two days. Only a slight reduction of prothrombin and proconvertin was found after these doses. The experiment was repeated with another cow, giving the PID as a 3 per cent solution intravenously in doses of 6 g every 6th hour. The results are shown in figure 1. The astonishing findings were that prothrombin in this species was reduced somewhat more than was proconvertin, and that the reduction after this treatment was too small to bring about any change in the thromboplastin time. On the basis of these findings, and for economical reasons, these experiments were discontinued.

Then a dog weighing 18 kg was fed 500 mg PID after 0, 10, and 20 hours: and the thromboplastin time, the activities of prothrombin, proconvertin, and proaccelerin were followed. The results

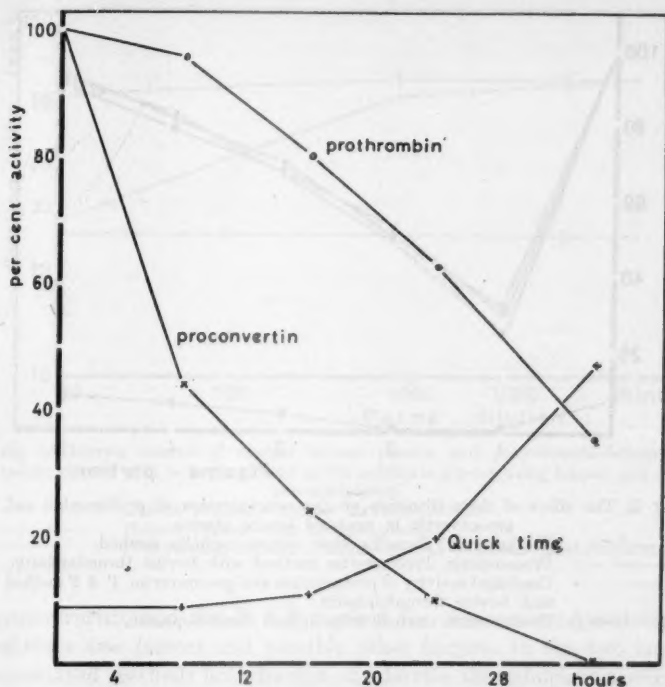


Fig. 2. The effect on the prothrombin and proconvertin level in plasma from a dog given phenylindanedione perorally.

The dog was fed 500 mg phenylindanedione after 0, 10, and 20 hours.

are shown in figure 2. A fairly good separation of prothrombin and proconvertin was obtained in this species. The animal was bled after 32 hours, and the plasma used as a substrate plasma for proconvertin assay. The dilution curve showed about the same slope as the proconvertin-deficient human plasma, but part of this was due to an influence of the prothrombin in the test plasma, as would also be expected from the low prothrombin content of the dog PID-plasma. Although this plasma was not entirely satisfactory, a usable substrate plasma might be prepared in this way.

B. Differential adsorption. Such experiments were not performed because of unsatisfactory results by other workers (see HJORT 1957, p. 36).

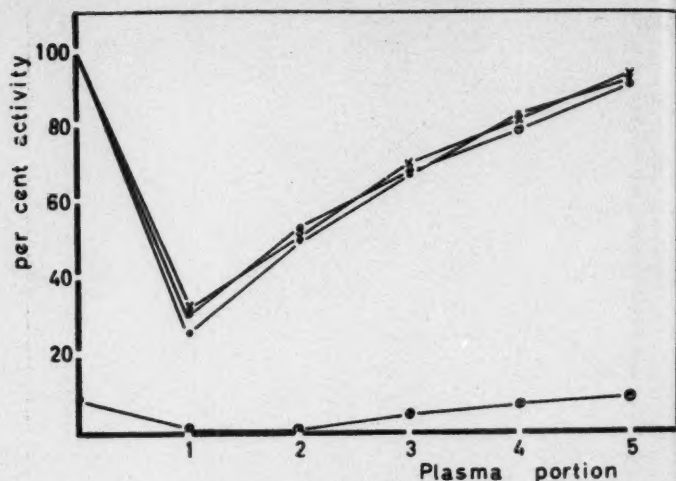


Fig. 3. The effect of Seitz filtration on the concentration of prothrombin and proconvertin in oxalated bovine plasma.

- x — x Prothrombin. Russell's viper venom-cephalin method.
- o — o Proconvertin. Proconvertin method with bovine thromboplastin.
- — • Combined activity of prothrombin and proconvertin. P & P method with bovine thromboplastin.
- ⊙ — ⊙ Proconvertin method with human thromboplastin.

C. *Filtration experiments.* Several workers have claimed that proconvertin can be separated from prothrombin by Seitz filtration of bovine plasma (OWREN and BJERKELUND 1949, DUCKERT, LOELIGER and KOLLER 1951, KOLLER, LOELIGER and DUCKERT 1951, AAS 1952, DE NICOLA 1953 and many others). However, a 20 per cent Seitz filtered ox plasma gave a horizontal standard curve with ox thromboplastin, and ox plasma as the test plasma, while with human thromboplastin and human test plasma, the same substrate plasma gave a standard curve with a reasonable slope. This discrepancy prompted an investigation into the reliability of Seitz filtration to separate prothrombin from proconvertin.

Oxalated plasma was filtered through 20 per cent asbestos (diameter 12 cm, pressure 0.5 kg per sq. cm) and collected in 100 ml portions. The following methods were used to follow the changes in the plasma during filtration: The Russell's viper venom-cephalin method for prothrombin, the "specific" method for

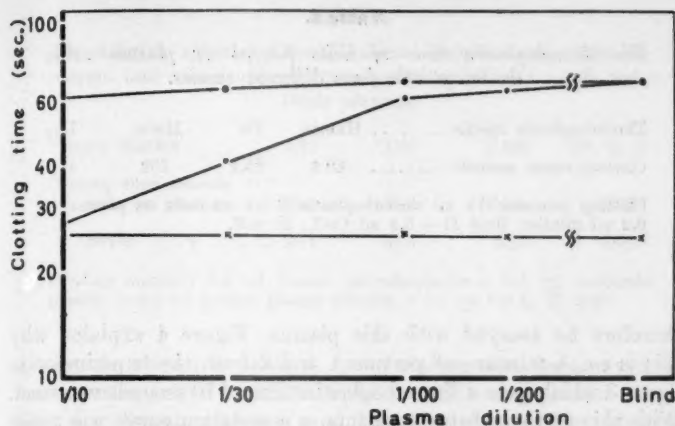


Fig. 4. Dilution curves of normal human plasma and proconvertin-deficient human plasma with Seitz ox plasma as the substrate plasma using human and ox thromboplastin.

- — Human plasma — human thromboplastin.
- × — Human plasma — ox thromboplastin.
- — Proconvertin-deficient human plasma — human thromboplastin.

proconvertin, and the P & P method for the combined activity of these two factors and possible other factors. In the two last mentioned methods both human and bovine thromboplastin were used. The results are shown in figure 3. It is evident from the curves with bovine thromboplastin that Seitz filtration did not separate prothrombin and proconvertin. When tested with human thromboplastin, however, a separation was apparently obtained. This apparent separation, obtained with human thromboplastin, is the basis for the widely accepted view that prothrombin and proconvertin can be separated in this manner. The results with the P & P method closely followed those for prothrombin and proconvertin. Since this method is supposed to measure all adsorbable factors partaking in the extrinsic system, it is reasonable to believe that Seitz filtration reduces all these factors to the same degree.

Can the Seitz plasma nevertheless be used as a substrate plasma for the assay of proconvertin? Comparison of the results obtained with this plasma and with the proconvertin-deficient human plasma both in this and other laboratories has shown that the results correlate fairly well. Proconvertin from human source can

Table 4.

The thromboplastin time of Seitz filtered ox plasma using thromboplastin from different species.

Thromboplastin species	Human	Ox	Horse	Dog
Clotting times, seconds	69.8	25.2	102	41.2
Clotting mixture: 0.2 ml thromboplastin + 0.2 ml Seitz ox plasma + 0.2 ml dilution fluid II + 0.2 ml CaCl_2 , 25 mM.				

therefore be assayed with this plasma. Figure 4 explains why this is so. A mixture of portion 1 and 2 from the experiment in figure 3 which gave a thromboplastin time of 70 seconds was used. With this as the substrate plasma, a correlation curve was made with progressive dilutions of normal human plasma. With human thromboplastin a correlation curve similar to that with proconvertin-deficient human plasma as substrate plasma was found (not illustrated in fig. 4), while with bovine thromboplastin no influence on the clotting times was observed. This indicates that the human thromboplastin reacts preferably with the proconvertin in the human test plasma, as opposed to the bovine thromboplastin, which reacts preferably with the proconvertin in the substrate plasma. Thus, the reaction between human thromboplastin and human proconvertin runs fairly independently of the proconvertin in the substrate plasma. That it is chiefly proconvertin which is measured, can be concluded from the upper curve in fig. 4, which represents the corresponding dilution curve with proconvertin-deficient human plasma as test plasma. Only an insignificant influence from prothrombin in the test plasma is seen with these dilutions. In spite of the findings reported, therefore, Seitz filtered ox plasma may be used as substrate plasma for the assay of human proconvertin.

Can proconvertin activity of other species also be assayed with Seitz filtered ox plasma? According to the results found in figures 3 and 4, it is clear that bovine proconvertin cannot be estimated with this plasma. For the other species, this question can simply be answered by testing the buffer time with the thromboplastin of the species concerned. The longer this time, the less is the affinity of bovine proconvertin for the thromboplastin in question, and the better can the Seitz plasma serve as substrate plasma for that

Table 5.

The dilution effect of normal human plasma using Seitz ox plasma and mixed adsorbed/unadsorbed ox plasma as the substrate plasmas.

Plasma dilution	1/10	1/30	1/100	Dil. fl. II
Clotting time seconds				
Seitz ox plasma	25.8	36.4	54.8	69.0
Mixed "	26.4	37.5	54.0	68.3

Clotting mixture: 0.2 ml human thromboplastin + 0.2 ml substrate plasma + 0.2 ml human plasma dilution + 0.2 ml CaCl_2 , 25 mM.

species. As shown in table 4, horse plasma may be assayed for proconvertin activity with this plasma, while dog plasma cannot when dog thromboplastin is used. However, since human thromboplastin reacts easily with dog proconvertin, the proconvertin activity in dog plasma may be assayed with Seitz filtered ox plasma using human thromboplastin.

Attempts to find a suitable substrate plasma for the bovine species failed. Both horse and dog plasma show a high proconvertin activity, and as can be seen from tables 1 and 2, ox thromboplastin reacts fairly well with the proconvertin of these species. In horse plasma proaccelerin is also reduced by Seitz filtration. None of these plasmas, therefore, yielded a satisfactory substrate plasma.

D. *Substrate plasma made by mixing normal and adsorbed ox plasma.* Because of the results with Seitz filtration, it should be possible to prepare a substrate plasma simply by mixing adsorbed and unadsorbed bovine plasma in such a proportion that the prothrombin and proconvertin content equals that of Seitz filtered plasma. Oxalated adsorbed bovine plasma was mixed with non-adsorbed bovine "silicone" plasma to give the same thromboplastin time as the Seitz filtered sample (about 70 seconds), which in this case was obtained by mixing 2/3 of adsorbed and 1/3 of unadsorbed plasma. This proportion, however, varies with the plasma level of prothrombin and proconvertin. In my opinion it is more correct to mix to a given thromboplastin time than to use a constant proportion. The same principle is true also for Seitz filtration, because it is impossible to reproduce the filtration procedure exactly, which results in a variation of the filtered samples. By subsampling the filtrate, and mixing to a given

Table 6.

The activity of proconvertin in different species as measured in the one-stage proconvertin assay using proconvertin-deficient human plasma with accelerin added.

Species	No. of animals	"Silicone" plasma		"Activated" plasma	
		\bar{m}	Range	\bar{m}	Range
Human	10	100	78—117	355	330—390
Ox	9	18	14—24	60	50—78
Horse	8	250	220—295	300	285—325
Dog	9	60	45—72	185	170—215

The thromboplastin used was homologous to the test plasma, and the figures refer to per cent in relation to normal human plasma activity.

thromboplastin time, this variation is largely avoided. — In table 5 the dilution effect of normal human plasma are compared, using the mixed plasma and the Seitz filtered plasma as substrate plasmas. The results show that a substrate plasma may be prepared by either of these methods.

There is, however, a serious drawback with both of the Seitz filtered and the mixed adsorbed/unadsorbed substrate plasmas: they are both unstable. The buffer time decreases during storage or on repeated freezings and thawings due to activation of the remaining proconvertin; and if the proconvertin is activated to a certain extent the plasma cannot be used. This can be illustrated by mixing experiments with normal adsorbed, and glass activated ox plasma; or glass activation of Seitz plasma, and the finding is easily explained by the difference in reactivity of human thromboplastin to ox plasma before and after activation (table 9). By mixing one part of normal, non-activated and 2 parts of adsorbed ox plasma, the human thromboplastin time was 78.5 seconds. To obtain the same time when activated plasma was used instead of non-activated, however, only one part to seven parts of the adsorbed was needed. The latter mixture cannot, of course, be used because of its low prothrombin content. Furthermore, if a Seitz plasma with a human thromboplastin time of 78 seconds was activated with Speedex, the time decreased to 31 seconds.

It is possible to stabilize these plasmas by adding small amounts of heparin immediately after preparation. Heparin blocks the activation of proconvertin according to RAPAPORT, AAS and OWREN (1955), and small amounts do not influence tests in which

Table 7.

The increase in activity of different proconvertins after exposure to glass particles.

Plasma	Human	Ox	Horse	Dog
Ratio: Activity after/activity before particles exposure to glass	3.5	3.3	1.2	3.1

thromboplastin is used. Therefore, one unit of heparin per ml was added to the substrate plasmas before freezing, and storing in siliconized tubes.

3. The Activity of Proconvertin in Different Species as Measured in Human Proconvertin-Deficient Plasma.

It must be stressed that the activity measured in this manner cannot be relied upon to reflect the true proconvertin concentration. This is so because both thromboplastin, proconvertin, and their reaction product, convertin, vary qualitatively in different species. The activities were measured by the one-stage method using human proconvertin-deficient plasma as the substrate plasma, and with accelerin added. The thromboplastin was homologous to the test plasmas, which were drawn with careful silicone technique. The figures in table 6 are relative to normal human plasma activity. It is seen from the column for "silicone" plasma that the activity was low in ox plasma and high in horse plasma, the dog and man being in between.

4. Activation of Proconvertin in Plasmas from Different Species.

The "silicone" plasmas from the preceding experiment were also assayed for proconvertin activity after activation with 1/4 volume of Speedex. The results are shown in the right column in table 6. On the basis of the figures in both columns in table 6, one can calculate the ratio "activated"/"silicone", which is then a measure of the property of the different proconvertins to increase its activity after glass activation. It is seen from table 7 that, except for the horse, the activity increased three—four times. In the horse only a limited activation occurred. The same was also found with the thromboplastin time and the P & P method when homologous thromboplastin was used. With heterologous thromboplastin,

Table 8.

The thromboplastin times of horse plasma with different thromboplastins before and after glass activation.

Thromboplastin species	Clotting times, seconds	
	"Silicone" plasma	"Activated" plasma
Human	14.2	10.5
Ox	17.5	12.5
Horse	10.1	9.8
Dog	19.0	16.0

Clotting mixture: 0.2 ml horse plasma + 0.2 ml thromboplastin + 0.2 ml CaCl₂, 25 mM.

however, the activation was about the same for horse as for the other species. This phenomenon is shown in table 8, giving the thromboplastin times for horse plasma before and after glass activation with different thromboplastins. In other plasmas, too, the activation ratio was different with different thromboplastins. Some results for ox plasma are presented in table 9, because they explain why Seitz filtered, or mixed adsorbed/unadsorbed ox plasma cannot be used as substrate plasmas for proconvertin assay after activation. It is seen that human thromboplastin reacted rapidly with the activated bovine plasma in contrast to the non-activated.

The phenomenon found with horse plasma and horse thromboplastin might explain the statement of QUICK that dog plasma contains no prothrombinogen, all the prothrombin being present in the active form. "Silicone" and "activated" dog plasma were, therefore, tested with rabbit brain thromboplastin (Difco) which

Table 9.

The reactivity of human thromboplastin to normal and glass activated ox plasma as compared with human plasma.

Plasma species	Clotting times, seconds	
	"Silicone" plasma	"Activated" plasma
Bovine	23.5	11.5
Human	14.6	10.2

Clotting mixture: 0.2 ml human thromboplastin + 0.2 ml plasma + 0.2 ml CaCl₂, optimal concentration.

Table 10.

The effect of glass activation of dog plasma on the thromboplastin time with rabbit brain thromboplastin.

Type of plasma	"Silicone"	"Activated"
Clotting times, seconds	9.2	5.8
Clotting mixture: 0.2 ml thromboplastin + 0.2 ml plasma + 0.2 ml CaCl_2 , 30 mM.		

is the type of thromboplastin used by QUICK. As is seen from table 10, the effect of glass activation was not less than in plasmas of other species. This finding does not, therefore, conform with the statement of QUICK.

5. Species Specificity of Proconvertin.

It is not possible to study the specificity of proconvertin *per se* since the available methods are based on the use of thromboplastin. It can thus only be studied through its reaction product with thromboplastin, *i. e.* convertin, which requires the use of the convertin assay method. The one-stage proconvertin method does not differentiate between the rate of convertin formation and the effect. Furthermore, purified reagents must be used for these studies. Serum, as used by BURSTEIN (1950) and MANN and HURN (1952), is unsatisfactory because it contains anticonvertin (HJORT 1957, p. 76) which might invalidate the results. The influence of the anticonvertin in human serum on different convertins is shown in figure 5. The human serum was oxalated with 1/5 volume of 2.5 per cent potassium oxalate and adsorbed with 100 mg of barium sulphate per ml of serum. The barium sulphate was removed by centrifugation, and the oxalate by dialysis overnight against four changes of saline. Thereafter it was recalcified to a final concentration of 2.5 mM. The convertins were made by incubating for 10 minutes 0.2 ml undiluted thromboplastin + 0.8 ml proconvertin solution of a concentration great enough to saturate the thromboplastin + 1.0 ml of 5 mM CaCl_2 . The convertin thus formed was incubated at 37° C with one ml of the serum, and the decrease in activity followed with the convertin method. The figure shows that the different convertins were inactivated at different rates. It is not easy to explain why the heterologous and

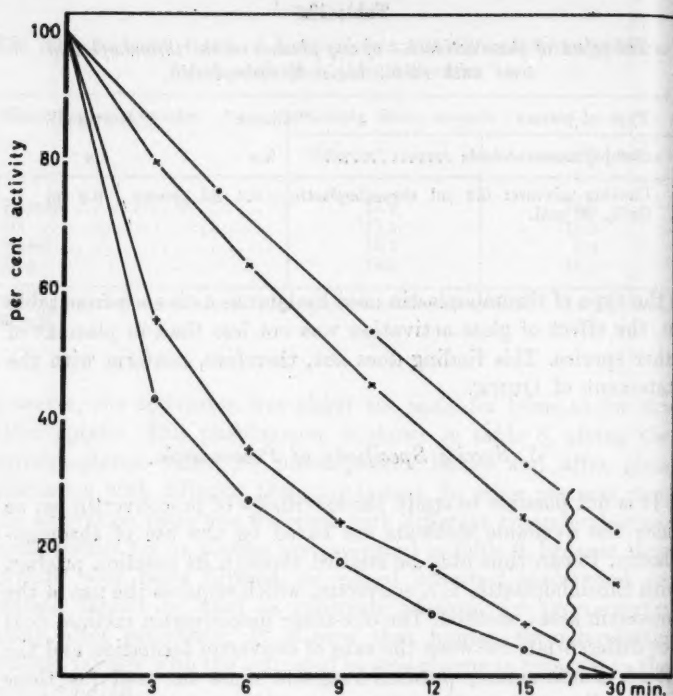


Fig. 5. The influence of anti-converting of human serum on different convertins

• — • Human proconvertin + human thromboplastin.
 × — × Human " + bovine "
 + — + Bovine " + bovine "
 o — o Horse " + horse "

semiheterologous convertins were inactivated faster than the homologous. Nevertheless, the experiment makes it clear that serum cannot be used for these studies.

To illustrate the species specificity of proconvertin a series of curves are presented in figure 6. The experiments were carried out as follows. The proconvertin reagent was titrated against its homologous thromboplastin, and a concentration chosen for the experiment which just saturated the amount of thromboplastin used. A $1/5$ dilution of the thromboplastin was used, and the titrated concentrations of proconvertin were $1/10$ and $1/2$ of the human and bovine stock preparation, respectively. Equal parts

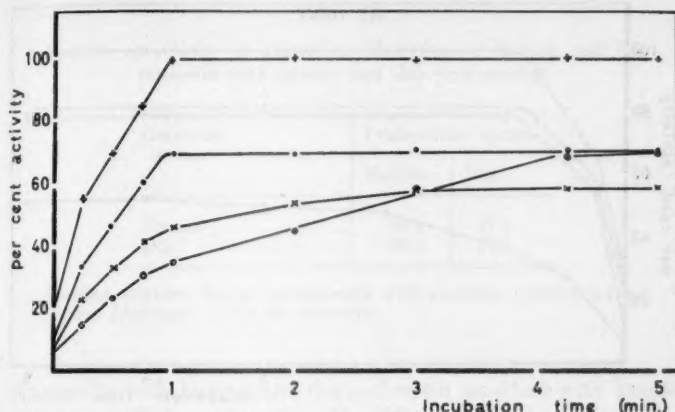


Fig. 6. Species specificity of thromboplastin and proconvertin: Differences in the reactivity patterns and in the effect of final activity.

+ — + Human proconvertin + human thromboplastin.
 • — • Bovine " + bovine "
 × — × Human " + bovine "
 o — o Bovine " + human "

of the dilute thromboplastin, proconvertin, and 7.5 mM CaCl_2 were then incubated in combinations as shown in figure 6, and the activity of the mixtures followed with the convertin method. Three different patterns of reactions between proconvertin and thromboplastin can be traced in this diagram. The first is the homologous reaction, which was fairly uniform for all species. The second is the reaction between bovine proconvertin and human thromboplastin, showing a slowly increasing activity which finally reached the same level as with bovine thromboplastin. The third is the reaction between human proconvertin and bovine thromboplastin, which showed a somewhat quicker initial increase in activity than the foregoing combination but never reached more than half the activity with human thromboplastin. These curves make it clear that not only thromboplastin but also proconvertin differ in different species, and that the specificity may exert its influence in two ways, viz. by variation in the rate with which the proconvertin and thromboplastin combine, and by variation in the activity of the final product.

That heterologous combinations might react similarly to homologous, is evident from figure 7, where it is seen that dog

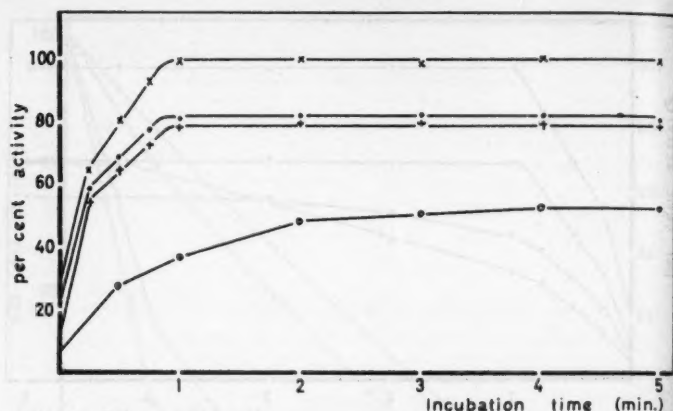


Fig. 7. Species specificity of thromboplastin and proconvertin: Example of superiority of a heterologous to a homologous thromboplastin.

x	—	x	Dog	proconvertin + human	thromboplastin.
•	—	•	Dog	» + dog	»
+	—	+	Human	» + human	»
o	—	o	Human	» + dog	»

proconvertin reacted even better with human than with dog thromboplastin. This conforms with the findings in table 1 and 2, which does also the result of the combination of human proconvertin and the same thromboplastins: dog thromboplastin reacted poorly with human proconvertin. The findings illustrated in figures 6 and 7 allow the following conclusion. In a one-stage proconvertin assay using thromboplastin from another species than the proconvertin, the activity measured refers as well to the velocity of the reaction between these factors as to the concentration of proconvertin. Homologous thromboplastin, therefore, should be used.

6. Species Specificity of Convertin.

Convertin was prepared from purified reagents of different species in the usual way. The strength of the preparations was adjusted to give the same clotting time with purified human prothrombin, and then tested with prothrombin purified from the other species. The purified prothrombins were, on beforehand, adjusted to the same "concentration" by means of the three-stage prothrombin assay using homologous reagents, and added a constant amount of accelerin. As an example, table 11 shows

Table 11.

Species specificity of convertin: Matching of human and dog convertin with human and dog prothrombin.

Convertin species	Prothrombin species	
	Human	Dog
Human	35.0	47.2
Dog	35.3	27.5

Clotting mixture: 0.2 ml prothrombin with accelerin added + 0.2 ml bovine fibrinogen + 0.2 ml convertin.

the results with human and dog convertin matched with human and dog purified prothrombin. The differences in clotting times in the horizontal columns indicate that convertin does not convert different species prothrombin at the same rate. This might be caused by species specificity of both convertin and prothrombin. That convertin per se showed species specificity is obvious from the vertical columns, since the two convertins gave the same clotting times with human prothrombin, but not with dog prothrombin. The convertins always gave the shortest clotting times with the homologous prothrombin.

Discussion.

Recent work has revealed new factors which probably take part in both the intrinsic and the extrinsic clotting system, the Prower factor (BERGSAGEL 1955) and the Stuart factor (GRAHAM and HOGGIE 1956). These factors might therefore increase the complexity of the species specificity of the thromboplastin-proconvertin reaction. It has not been possible, however, to consider their influence since we lack methods for the assay. The factors did probably not influence the results with the proconvertin method used here, because the substrate plasma was a pure proconvertin-deficient plasma with normal Russell's viper venom time. It is therefore thought that the results obtained are valid for proconvertin.

The experiments with administration of phenylindanedione to cows in order to produce a substrate plasma for the assay of

proconvertin, were given up chiefly for economical reasons, and they must be regarded as preliminary. The comparatively small effect of the doses given intravenously indicate that large doses of this drug are needed to bring about an appreciable reduction of prothrombin and proconvertin in this species. Possible explanations are that there are species differences in the resistance to different dicumarol-like drugs, or, that sweet clover disease is not caused only by the ingestion of dicumarol, but also by the derangement of the vitamin-K producing microorganisms in the rumen. — Similar experiments were not performed with the horse, because of the high proconvertin activity in this species, and the costs. In dog it was possible to produce a usable substrate plasma by the administration of PID, but it cannot be recommended as a good method.

The filtration experiments showed that proconvertin cannot be separated from prothrombin by Seitz filtration (fig. 3). This may be regarded as proven by the finding that a practically identical substrate plasma could be prepared by mixing normal and adsorbed ox plasma (table 5). The widely accepted view that such a separation is possible, is an illusion due to the ignorance of the species specificity of thromboplastin (fig. 4). It should be mentioned that AAS (1952) was unable to separate proconvertin from prothrombin in human plasma, but succeeded with bovine plasma. This can be explained by his use of human thromboplastin. The separation has been questioned by FELL, JOHNSON and SEEGER (1954), but their experimental evidence was not conclusive.

In spite of these findings, Seitz filtered, or mixed normal/adsorbed ox plasma may be used as a substrate plasma for the assay of proconvertin in human plasma using human thromboplastin, in horse plasma using horse thromboplastin, and in dog plasma using human thromboplastin. This is so because bovine proconvertin has little affinity to these thromboplastins (table 4), and the proconvertin-thromboplastin reaction of the test plasma can therefore run fairly independently of the proconvertin in the substrate plasma. — Another problem which, as far as can be seen, has not been observed previously, is the instability of Seitz plasma (and also the mixed normal/adsorbed) due to activation when stored in ordinary glass. This makes the plasma unsuited as a substrate plasma (cfr. prg. 2 D.). The addition of 0.5—1.0 unit of heparin per ml of the substrate plasma, and

the use of siliconized tubes for storage are measures recommended to minimize the activation.

Quantitative comparisons of proconvertin in different species have been made by DEUTSCH and SCHADEN (1953), DE NICOLA (1953) and others. According to the findings concerning the species specificity of the proconvertin-thromboplastin reaction (figs. 6 and 7) it is clear that such comparisons are illusory, because any difference can as well be qualitative as quantitative. The results will depend much on the species origin of the reagents used, and the value of such comparisons is thus limited. The statement of DE NICOLA (1953), that the concentration of proconvertin is the decisive factor for the thromboplastin time, should also be mentioned in this connection. It was based on a comparison between the thromboplastin time and proconvertin "concentration" of different species using one and the same thromboplastin, and the one-stage proconvertin assay. In light of the results in this paper (figs. 6 and 7), he therefore measured the reactivity of the particular thromboplastin to the different proconvertins in both methods and the results should accordingly be parallel. Thus his statement refers to reactivity rather than to concentration, and is then in line with the results reported here (confer table 9; also that one part of activated plasma to seven parts of adsorbed plasma gave the same clotting time as one part of non-activated to two parts of adsorbed plasma).

MANN and HURN (1952) found that after incubation of thromboplastin with its homologous serum, the species specificity was largely eliminated. According to the results in this paper it is not eliminated (table 11). Their experiments might be criticized because they used serum and not purified proconvertin, and because they tested the convertin activity in plasmas from different species with widely varying proaccelerin concentration, which influences the results. This can be avoided by using prothrombin purified from the different species, with a constant amount of accelerin added.

The results with dog plasma seen in table 6, do not conform with the statement of QUICK and HUSSEY (1951) that dog plasma contains no prothrombinogen (proconvertin). It was thought that this might be explained by the same phenomenon as with horse thromboplastin and horse plasma (table 8) showing little difference in the thromboplastin time before and after glass activation. However, the experiment with rabbit brain thromboplastin and

dog plasma (table 10) did not support this assumption, and the explanation for this discrepancy is obscure.

Summary and Conclusions.

1. The species specificity of thromboplastin was confirmed. Different thromboplastins reacted differently with various plasmas before and after glass activation.
2. Proconvertin and its reaction product with thromboplastin, convertin, also showed qualitative differences in different species.
3. Due to these findings a quantitative comparison of proconvertin concentration in different species cannot be done with present-day methods.
4. The proconvertin-deficient human plasma is recommended as the substrate plasma for the assay of proconvertin.
5. Prothrombin and proconvertin cannot be separated in bovine plasma by Seitz filtration as previously assumed. In spite of this, Seitz ox plasma may be used as a substrate plasma for the assay of proconvertin in certain species.
6. Since the effect of Seitz filtration was only a reduction of the prothrombin and proconvertin concentration to the same extent, a similar substrate plasma could be prepared by mixing non-activated ox plasma and adsorbed ox plasma to give a human thromboplastin time of 70—80 seconds.
7. The Seitz ox plasma and the mixed non-activated/adsorbed ox plasma can be used as substrate plasmas only when care is taken to avoid activation of the remaining proconvertin.

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Life Cycle of Granulocytes and Lymphocytes Determined by Making Use of ^{59}Fe Labelled Haemin as a Tracer.

By

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The white blood cells contain iron (MACALLUM 1891). The form in which all this iron is present is not known. Enzymes containing iron were found by many authors: AGNER (1941) found in granulocytes isolated from aseptic abscesses a large amount of verdo-peroxidase. In both granulocytes and lymphocytes cytochrome C is assumed to be present. According to SIMMONS and EVERETT (1954) 10 % of the iron content of leucocytes is present in a ferritin-like compound. The presence of cytochromoxidase was observed in the lymphocytes as well (WACHSTEIN 1955).

We wished to investigate if and to what extent iron atoms of the leucocytes are renewed.

It is possible to approach this problem by comparing the pattern of incorporation of radioactive iron into the haemins of leucocytes with the incorporation of ^{32}P into DNA of these cells as determined by OTTESEN (1954) and other investigators. A turnover of DNAP was found by these to be absent, and should this also be the case for the iron atoms of the haemins of leucocytes, we ought to arrive at the same result for the life cycle of leucocytes as did the above mentioned authors. We studied incorporation of ^{59}Fe both in haemin fractions of lymphocytes and of granulocytes.

The haemins discussed in the paper are the haemin fractions only which are extractable with HCl-acetone.

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Experimental Methods.

The experiments were performed on 16 healthy adult rabbits, weighing 3–4 kg, showing normal white cell and differential counts. In view of the minute iron content of leucocytes, a rather large amount of cells was necessary for each determination. A new animal therefore had to be used for each experiment and the total blood collected. ^{59}Fe labelled leucocytes were obtained by the following procedure. 15–20 ml of blood secured by cardiac puncture from each rabbit were centrifuged and the erythrocytes, suspended in isotonic saline, were reinjected at once. In order to label the iron of the β_1 -globulin of the plasma 10 ml of plasma obtained were incubated for 20 min. at 37° with 7–10 μg of labelled iron containing iron as citrate and of a specific activity of about 1.5 μC per μg of iron. The plasma was then reinjected intravenously.

Two samples of the labelled plasma were taken and used as standards in the calculation of the total activity injected. The animals were sacrificed at different intervals between 6 hours and 23 days after injection. The blood was collected under urethane narcosis by cannulation of one carotid using heparin as anticoagulant. About 100 ml of blood were obtained.

The separation of lymphocytes and granulocytes was carried out according to the method of OTTESEN. In order to avoid the contamination of lymphocytes by platelets the blood was centrifuged for 10 min. at 150 g, the plasma was discarded and the cells resuspended in isotonic saline. Both white cell fraction obtained at the end of this procedure contained 10 to 15 % of R. B. C. While the presence of a small percentage of erythrocytes was permissible in OTTESEN's experiments, since the red corpuscles do not contain DNA, we had to prepare leucocytes entirely free from erythrocytes. Washing the white blood corpuscle fraction twice with saline containing 0.16 % saponin sufficed to remove the contaminating erythrocytes as revealed by microscopic control. We then rinsed the fraction with saline until the spectrum of haemoglobin could no longer be observed. To show that the ^{59}Fe found in the lymphocytes did not enter these via the radioactive iron of haemolyzed erythrocytes we carried out the following experiment. Inactive W. B. C. were added to active erythrocytes which were then haemolyzed. The W. B. C. purified as described above were found to be entirely inactive. The lymphocyte fraction was almost pure, impurities amounting to not more than 5–10 % and consisting of monocytes, granulocytes and a few platelets. 95 % of the granulocytes were neutrophils.

From each animal samples of bone marrow, lymph nodes and thymus were taken, homogenized in saline and washed twice with a saponine solution in order to haemolyze the erythrocytes present. The haemins were extracted with acetone containing 1 % HCl and separated from the precipitated proteins by filtration. The acetone was carefully evaporated under gentle suction, the haemins dissolved in chloroform

and purified by repeated treatment with 1 N HCl in a separator funnel. The chloroform was then destroyed by wet ashing and its iron content brought into solution. One known fraction of the solution obtained was applied in the determination of its iron content by the sulphosalicylic acid method of LORBER (1927). Another known fraction was used in activity measurements. These were carried out as described by AGNER, BONNICHSEN and HEVESY (1954). From the iron content and its activity the relative specific activity was calculated. The bulk of precipitated proteins remaining after extraction of the haemin of each sample was treated in the same way to obtain the specific activity of with acetone not extracted iron.

In experiments in which labelled leucocytes were transfused the procedure applied was the following. The donor animals were injected intraperitoneally with 30 μ C of ^{59}Fe and sacrificed after 24 hours. The leucocytes were obtained by setting the R. B. C. with dextran¹ (m. w. 179,000). A mixture of equal volumes of blood and of a solution of 5 % dextran in isotonic saline was found to give the best result. The leucocytes were centrifuged for 15 min. at 150 g, the supernatant discarded and the cells resuspended in 20 ml saline injected intravenously into another rabbit. No further attempt to purify the leucocytes was made in order to avoid damage of the cells. To determine the fraction of activity due to the presence of erythrocytes, the activity of the suspension to be injected was determined prior and after removal of erythrocytes.

The receptor rabbits were killed after 1 and 3 days. The white blood corpuscles were separated and treated as described above.

Results of Specific Activity Determination.

a) *Granulocytes*. — The change of the specific activity of haemin iron with time is seen in Figure 1. It increases for the first 4–5 days and then decreases. The decreasing part of the curve indicates a rapid process prevailing for about 6 days and a slower process after that date. The granulocytes have a slight ^{59}Fe content even after the lapse of 23 days. No samples were taken after that date, but by extrapolating the curve we obtained the result that no ^{59}Fe is left in the granulocytes after the lapse of about 26 days. This is the time interval after which in OTTESEN's experiments ^{32}P disappeared from DNA of with labelled phosphate injected human subjects. We can therefore conclude that in the granulocytes the haemin iron is not in a dynamic state. The iron atoms are incorporated into the haemin molecule during its formation and remain until the granulocyte ends its life cycle.

¹ The dextran was a kind gift of Pharmacia in Uppsala.

Thus the labelling of the haemin iron results in a permanent labelling of the granulocyte. We can correspondingly determine the average life span of the granulocytes by following up their haemin ^{59}Fe content.

To arrive at the life span of granulocytes we divide the time of the experiment in n equal intervals. The specific activity of the precursor for each interval is $f(\frac{1}{2})$, $f(1\frac{1}{2})$, $f(2\frac{1}{2})$ $f(n-1\frac{1}{2})$. If these intervals are rather long, say 24 hours, then in view of the rapid clearance of the injected iron atoms from the plasma, it suffices to consider $f(\frac{1}{2})$ only and the remaining magnitudes can be disregarded. Correspondingly all ^{59}Fe in the granulocytes was incorporated in the course of the day. From this follows that the fraction of the first day production of granulocytes having the age between 0 and 1, 1 and 2, 2 and

$$3 \dots n-1 \text{ and } n \text{ days amount to } \varphi(\frac{1}{2}) = \frac{g_1}{f(\frac{1}{2})}, \quad \varphi(1\frac{1}{2}) = \frac{g_2}{f(\frac{1}{2})}, \quad \varphi(2\frac{1}{2}) = \frac{g_3}{f(\frac{1}{2})} \dots \varphi(n-1\frac{1}{2}) = \frac{g_n}{f(\frac{1}{2})} \text{ where } g_1, g_2, g_3 \dots$$

.. g_n are the specific activity of the haemin iron in the granulocytes in the 1st, 2nd, 3rd n^{th} day of the experiment.

The calculation of the $f_{(t)}$ values is frustrated by the fact that the specific activity of the precursor of the haemin iron is not known. However, from the slope of the curve seen in Fig. 1 it is possible to calculate the average life span (T) by making use of OTTESEN's equation

$$T = \frac{\int_0^{\infty} t\varphi(t)dt}{\int_0^{\infty} \varphi(t)dt}$$

in which 2 terms containing the specific activity of the precursor cancel themselves.

The average life span of granulocytes calculated making use of the data supplied by Fig. 1 works out to be 8.8 days. If we calculate the half life time as indicated both by the rapid and

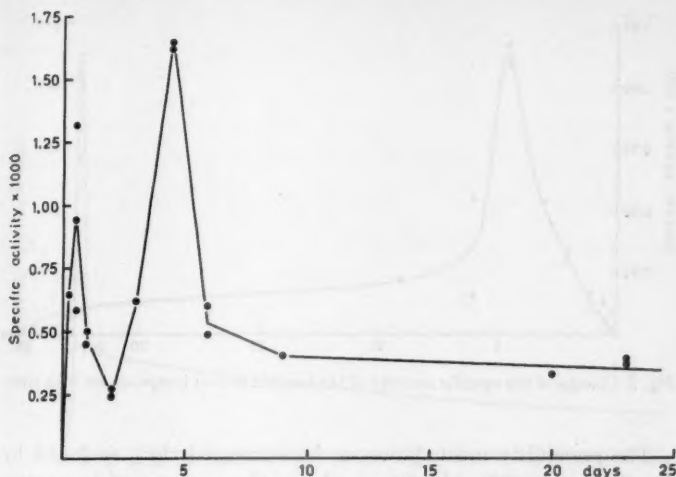


Fig. 1. Change of the specific activity of the haemin iron of granulocytes with time.

the slow component of the decreasing part of the curve we arrive at the result that the granulocytes mixture contains 2 kinds of particles having a different life span: 4.4 and 13.2 days.

b) *Lymphocytes*. — The curve which indicates the change of specific activity for lymphocyte haemin iron with time is seen in Fig. 2. The specific activity increases very steeply in the first 12 hours; decreases then until the 2nd day. A second increase is then observed which reaches its maximum at 4.5 days. On the 23rd day the specific activity present in the lymphocytes is still rather high and is decreasing at a very low rate only. These results indicate the presence of 2 lymphocyte fractions of different lifetime.

Fig. 2 is very similar to that seen in OTTESEN's paper except for the first peak at 12 hours which is not present in OTTESEN's figure. A possible explanation of this difference is the following. Lymphocytes incorporate ^{32}P in DNA during an early stage of their formation, while ^{59}Fe is partly incorporated shortly before their entrance into the circulation or even after that date. The last mentioned incorporation is not due to a renewal process but to an additional formation of haemin as indicated by the trend of the curve seen in Fig. 2.

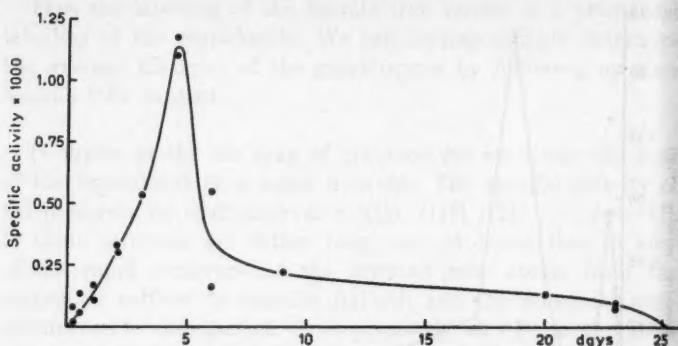


Fig. 2. Change of the specific activity of the haemin iron of lymphocytes with time.

The possibility must, however, be envisaged that, as found by FICHTELIUS (1957), bleeding leads to the passage of leucocytes stored in the lymphoid organs into the circulation, a process which may lead to the appearance of the second maximum indicated by Fig. 2.

To obtain each point indicated in Figs. 1 and 2 we had to collect the total blood content of a rabbit. Each point is thus obtained by investigating another rabbit and a statistical treatment of the result obtained in the usual way is therefore hardly feasible. But as seen in Figs. 1 and 2 when the W. B. C. of 2 rabbits were investigated after the lapse of the same time very similar values were obtained.

Comparing the integrals *i. e.* the areas indicated by the curve for 0—10 days time and then from 10 to 300 days, we arrive at the result that 10 % of the lymphocytes are short living, the rest long living. By carrying out this calculation we make the assumption that the specific activity of the haemin iron gets negligible after the lapse of 300 days. The mean life span of the short living lymphocytes evaluated graphically as done by BUSH, BERLIN, YENSEN, BRILL, CARTWRIGHT and WINTROBE (1955) for erythrocytes, works out to be about 3 days, while for the long living fraction a life time of 145 days is obtained. As was to be expected the first peak shown by the curve of Fig. 2 is almost absent in Fig. 3. The decrease in the specific activity of plasma iron was taken into consideration for the reason stated above when drawing Fig. 3, not however when drawing Fig. 2.

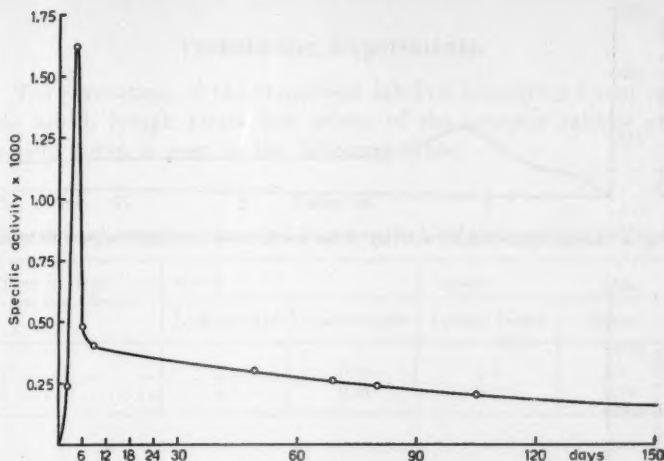


Fig. 3. Change of the specific activity of the haemin iron of lymphocytes plotted against time. Values corrected for the change of the specific activity of the plasma iron with time.

An alternative interpretation of the second part of the curve depicted in Fig. 3 is that the "long living" lymphocytes are destroyed in the same organs in which they are formed and we are faced with a renewal process which prevents the calculation of life time of lymphocytes for the same reason for which the calculation of the life time of erythrocytes cannot be effected from ^{59}Fe data.

A third hypothesis can be taken into consideration. The observed data could be explained on the basis of a large extra vascular pool of lymphocytes equilibrating only slowly with the peripheral circulation. However this interpretation involves the assumption that the same cell passes repeatedly from the blood to the tissues and back into the blood again. This assumption cannot be maintained in view of the results of DRINKER and YOFFEY (1941). These authors found that only 1 every 32 lymphocytes reenters the blood stream. By putting forward the above consideration we assumed all lymphocytes to behave equally. As we arrive at almost the same result for the life cycle of the long living lymphocytes as does OTTESEN, the first mentioned interpretation is possibly the correct one.

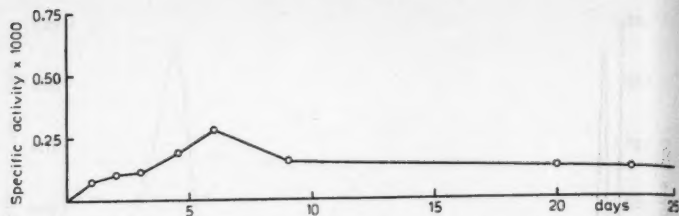


Fig. 4. Change of the specific activity of the haemin iron of lymph nodes with time.

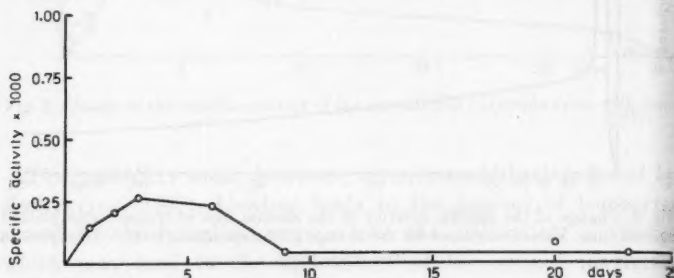


Fig. 5. Change of the specific activity of the haemin iron of the thymus with time.

Lymphoid organs. — The organs in which the production of lymphocytes takes place are lymph nodes, spleen, thymus and bone marrow. The spleen being very small in the rabbit its share in the production of lymphocytes is presumably a restricted one. A study of the ^{59}Fe incorporation into haemins of lymphocytes in the bone marrow is hardly feasible as only a very small fraction of the ^{59}Fe of the bone marrow is incorporated into lymphocytes. We could, however, study the incorporation of ^{59}Fe into the haemin fraction of the lymph nodes and in those of the thymus. The results obtained are seen in Figs. 4 and 5. In the lymph nodes the maximum specific activity is reached after 6 days, then it begins to decrease at an appreciable rate until the 9th day, a slow further decrease taking place after that date.

In the thymus the maximum specific activity is reached after 3 days. The decrease is very marked, so that after 9 days the specific activity is very low and remains constant until the 23rd day.

A comparison of the two curves (Fig. 4 and 5) suggests that the thymus produces primarily short living lymphocytes.

Transfusion Experiments.

The percentage of the transfused labelled leucocytes found in the blood, lymph nodes and spleen of the receptor rabbits at various times is seen in the following table.

Table 1.

Percentage of transfused leucocytes found in the blood and organs of rabbits.

Time in days after transfusion	Blood		Organs	
	Lymphocytes	Granulocytes	Lymph Nodes	Spleen
1	1.6	0.25	1.3	2.5
3	0.3	0.38	0.118	0.24

Already 1 day after the transfusion a very small amount of the injected leucocytes is present in the blood and in the investigated tissue fractions. After 3 days the number of the transfused lymphocytes both in the blood and in the lymphoid tissue is further decreased while the number of injected granulocytes hardly changes.

Discussion.

Granulocytes. — We have seen that the specific activity of haemin iron of granulocytes increases gradually from the first day reaching a maximum after 4.5 days. No similar result was found by OTTESEN who followed the change of the specific activity of the phosphorus of DNA of granulocytes with time. This difference between OTTESEN's and our results is presumably due to the fact that while DNA is synthesized in an early stage of the formation of leucocytes and thus it takes days before its ^{32}P reaches the circulation, ^{59}Fe is incorporated into leucocytes in the various stages of their maturation and is correspondingly found soon after the start of the experiment in the circulating granulocytes.

As seen in Table 2 different authors applying different methods arrive at values for the life time of granulocytes which strongly differ. The life span values arrived at by making use of the transfusion technique (VAN DYKE and HUFF 1955) are much too

Table 2.
Life span of leucocytes.

Cell type	Author	Method	Species	Life span
<i>Granulocytes</i> a) normal	WEISKOTTEN	marrow injury	rabbit	3—4 days
	PONDER, SASLOW and SCHWEIZER	" "	rabbit	2—3 week
	VAN DYKE and HUFF	X-ray parabiosis	rat	23 min.
	OSGOOD, TIVEY, DAVISON, SEAMAN and LI	marrow culture	human	108 hours
	OTTESEN	³² P DNA	human	9 days
	present author	⁵⁹ Fe hemin	rabbit	8.8 days
b) leukemic (chronic)	OSGOOD et al.	³² P DNA	human	3 days
	WEISBERGER and LEVINE	³⁵ S L-cystine	human	slightly less than 13 days
<i>Lymphocytes</i> a) normal	ADAMS, SAUNDERS and LAWRENCE	lymph flow	cat	12 hours
	YOFFEY	" "	cat	12 hours
	SANDERS, FLOREY and BARNES	" "	rabbit and cat	6 hours
	REINHARDT	" "	rat	12 hours
	OTTESEN	³² P DNA	human	4 days
	present author	⁵⁹ Fe hemin	rabbit	ca. 160 days
	OSGOOD et al.	³² P DNA	human	3 days
	WEISBERGER and LEVINE	³⁵ S L-cystine	human	ca. 145 days
	MCCALL, SUTHER- LAND, EISENTRAU and LANZ	⁵¹ Cr	human	30 days
				much more than 13 days
<i>Leucocytes</i> all forms	KLINE and CLIFFTON	³² P DNA	human	13 days
	WEISBERGER and LEVINE	³⁵ S L-cystine	human	13 days
	LAWRENCE, ERVIN and WETRICH	cross circulation	cat	7—10 hours

short as the transfused granulocytes are soon destroyed in the receptor organism. In our experiments after the lapse of 1 day only 0.25 % of the transfused leucocytes was recovered.

The calculation of the life cycle (2 hr.) from the formation rate of the myeloid cells leads to much too low values for the life cycle of granulocytes as well, as the number of granulocytes present in the circulation represents only a small percentage of these particles present in the organism. OSGOOD (1954) estimates the number of stored granulocytes to be 60—80 times as large as that present in the peripheral blood, thus we have to multiply

the above mentioned life time (2 hr.) by 60—80 to arrive at a proper figure.

The presence of large granulocyte reserves in the organs is demonstrated by the results of CRADDOCK, ADAMS, PERRY, SKOOG and LAWRENCE (1955) as well. Presumably the bone marrow is storing a large part of leucocytes but the results of BIERMAN, KELLY, PETRAKIS, CORDES, FOSTER and LOSE (1951) suggest that a large number of leucocytes is stored in the lungs as well. They presented evidence that after injection of histamine a large part of granulocytes is removed from the circulation into the lungs in the first 30—60 seconds, returning later. The capacity of the lungs to remove white blood corpuscles from the circulation and to release them again was also demonstrated by AMBRUS, AMBRUS, PACKMANN, JOHNSON, BACK, CHERNICK and HARRISON (1953). The labelled leucocytes found by us to be present, for example, after the lapse of 3 days in the circulation may also have been stored temporarily in the lungs.

When labelling leucocytes *in vivo* and following the rate of their disappearance we can expect to arrive at correct values of their life-cycle as OTTESEN (1954) labelling the phosphorus of DNA finds 9 days for human granulocytes while we arrive labelling their haemin iron for the rabbit to 8.8 days, thus the same value.

As emphasized by SIERACKI (1955) the length of life of granulocytes is composed of a maturation phase which dates from the birth of the cell in the haemopoietic tissue until its release into the circulation, a second intravascular phase and of a third phase in which the granulocytes find refuge in the tissues. The two last mentioned phases may be repeated several times prior to the death of the cell.

The important role of granulocytes in emergency makes the necessity of large reserves of these cells easily understandable.

Lymphocytes. — The great difference in the life time values of lymphocytes found by different authors and stated in Table 2 is to be explained in a similar way as the strongly differing values obtained for the life cycle of granulocytes.

By blocking the lymphatic channels it was demonstrated that lymphocytes enter the circulation passing through the lymph stream (BLALOCK, ROBINSON, CUNNINGHAM and GRAY 1937). By counting the number of lymphocytes entering into the latter we arrive at the rate of their formation. The number of lymphocytes formed daily being 2—4 times larger than the number of

lymphocytes present in the circulation, the life time calculated from the above data, 6—12 hr., is clearly much too short.

Also for the lymphocytes it is a very great difference between the circulating and the total number of cells which includes the very large percentage stored in the organs. OSGOOD (1954) estimates the number of stored lymphocytes to be in the human 400 times larger than that of the circulating ones. In the rabbit organism in which less lymphoid tissue is present than in the human the above ratio can be estimated to be 100—200 (HELLMAN and WHITE 1930).

By studying the rate of disappearance of ^{32}P from the DNA of human lymphocytes OTTESEN arrived at the result that two lymphocyte populations exist, one having a life span of 4, the other of about 160 days. The life time of the latter is 100—200 times longer than the time spent in the circulation making out 12 hr. Our results fully corroborate those obtained by OTTESEN.

If all the lymphocytes produced in the lymph nodes were to enter the circulation we had to expect the haemins of the lymphocytes of the circulation and of the lymphoid tissue to have the same specific activity. The very much lower values found for the latter may be due to the fact that in the investigated lymphoid organs (lymph nodes and thymus) freed from erythrocytes other cell types beside the lymphocytes are present which contain haemin formed at a low rate, thus of low specific activity.

Summary.

In granulocytes and lymphocytes separated from the blood of the rabbit the incorporation of radioactive ^{59}Fe into the haemin fraction was investigated.

Evidence is presented that the iron is incorporated into the haemins during the formation of these cells and remains in them during their life time. From the change of the specific activity of the haemin iron with time the average life span of both lymphocytes and granulocytes can thus be calculated.

The mean age of the granulocytes was found to be 8.8 days. It is presumably composed of two populations of about the same frequency but varying life time, one having a life span of 4.4, the other of 13.2 days. The mean age of 8.8 days is identical with the result obtained by OTTESEN from ^{32}P data of DNA isolated from granulocytes.

A very appreciable part of lymphocytes has a life span amounting to 145 days; 10 % only having one of 3 days. An almost identical result was arrived at by OTTESEN in his DNA studies. A part of the lymphocytes entering the blood stream is still going on to incorporate haemin iron, thus to build up haemin.

Acknowledgment.

The author wishes to express his sincere gratitude to Professor G. de Hevesy for his stimulating interest and helpful suggestions throughout the work. His thanks are also due to Dr. G. von Ehrenstein and to Dr. L. F. Lamerton for helpful advice, to the Swedish Cancer Foundation and the Knut and Alice Wallenberg Foundation for their support of this investigation.

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Oxygen Dissociation Curves in Fish Blood.¹

By

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Fish blood shows certain peculiarities in the binding of oxygen which have not so far been described for mammalian blood. When CO_2 or acid is added to the blood, part of the hemoglobin may become completely unable to combine with oxygen, even at very high pressure, and in some species one obtains dissociation curves which are undulatory. We shall describe below the blood of a fish which displays both of these features, and we shall discuss briefly the binding of oxygen by hemoglobin in the light of some recently published high pressure dissociation curves.

It was discovered by ROOT (1931) that when the blood of tautog or toadfish was acidified with CO_2 or lactic acid it produced not simply a Bohr effect, *i. e.*, a lowering of the oxygen affinity, with full oxygenation still obtainable at high enough pressure but it apparently suffered a loss of oxygen capacity. The highest oxygen pressure used by ROOT was only that of air, but it was shown by GREEN and ROOT (1933) and by ROOT and IRVING (1941) that part of the hemoglobin remained unoxygenated even at near one atmosphere's PO_2 . It has long been believed that this "Root effect" is the direct chemical cause of the oxygen secretion in the swimbladder of fishes. This could be true, however, only if the dissociation pressure of oxygen produced by adding CO_2 or acid were greater than the oxygen pressure known to exist in the swimbladder of the fish. This prompted an investigation of the

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oxygen dissociation in the blood of a dozen deep sea fishes at various acidities and at pO_2 ranging from 0.2 to 140 atmospheres (SCHOLANDER and VAN DAM 1954). The result was that in some species the Root effect persisted at very high pressures, but in others full saturation was reached at a pressure much lower than that against which the fishes are known to secrete, which casts serious doubt on the Root effect as the cause of high pressure oxygen secretion.

Another interesting feature found in the whole blood of some fishes is that the oxygen dissociation curve at slight acidity or CO_2 tension is undulatory, with two portions concave to the abscissa. Such curves have been described for the toadfish (GREEN and ROOT 1933) and for tautog (ROOT and IRVING 1941). Undulating curves are striking in the blood of certain mollusks and crustaceans containing hemocyanin (REDFIELD and INGALLS 1933).

In our studies on deep sea marine fishes we came across one single species, the common silver hake (*Merluccius bilineatus*) which at pH 6 showed, like so many other species, a pronounced Root effect, i. e., the dissociation curve remained horizontal from 20 to 140 atmospheres' pO_2 at a saturation of 40 %. At a pH of about 7, however, the curve was conspicuously diphasic, with a plateau at 55–60 % saturation when the pO_2 was between 20 and 40 atmospheres. Full saturation was reached at near 100 atmospheres. With less acidity the plateau gradually vanished (Fig. 1).

It is of interest that among 30–40 high pressure curves which showed a Root effect there is no apparent tendency for the inactivation plateaus to cluster around even quarters, i. e., at 25, 50 and 75 % saturation. The curves may level off at any saturation from 30 % and up to 100 %. This situation cannot readily be derived from either the intermediate compound theory of ADAIR (1925), or from the heme-heme interaction theory of PAULING (1935), if we assume that our fish blood has four hemes per molecule of hemoglobin, such as indicated by the work of SVEDBERG and HEDENIUS (1934).

The multiple component theory was evolved by REDFIELD and INGALLS (1933), who showed that it applies very accurately to the oxygen combination with hemocyanin. The theory postulates a heterogeneous population of oxygen-combining molecules in the same blood, each component obeying the Hill equation. If each hemoglobin molecule contains four hemes, n may be taken

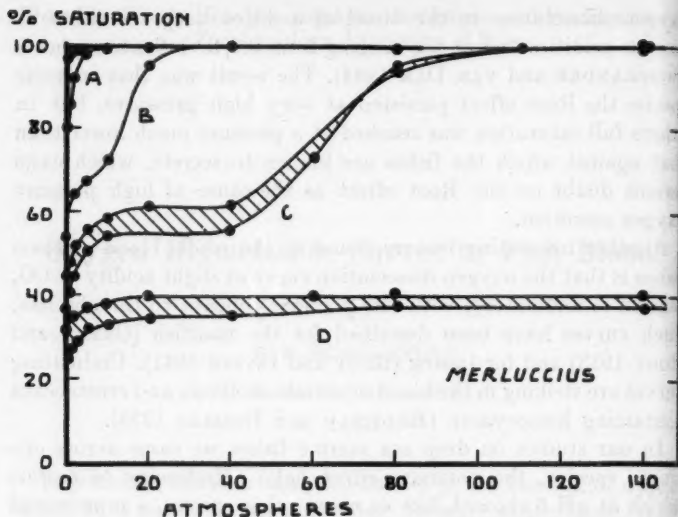


Fig. 1. Oxygen dissociation curves for the blood of silver hake (*Merluccius bilineatus*). Blood acidified with lactic acid. A corresponds to pH 7.7 C to 7.0, and D to 5.3.

as 1, 2, 3 or 4, K may be any number, and the fraction of each component (a) may be any number from 0 to 1. This of course represents a very considerable freedom in the choice of constants, and various authors have been able to construct curves which fit blood data from fish very closely, whether they are simple hyperbolic curves, sigmoid curves, curves with a Root effect, or undulating curves. Usually one need only postulate a mixture of two different components in order to produce a good fit to any given curve.

According to this theory each component would also produce plateaus determined by the number of inactivated hemes in the molecule, and in order to get a plateau in a four heme compound between even quarters at least two components would have to be present, each with a plateau at a different quarter, *e. g.*, one at 25 %, the other at 50 % saturation. This, again, means that any curve with a plateau between even quarters must be treated as the sum of two curves and cannot physically be regarded as a simple hyperbolic or exponential curve asymptotic to this plateau.

Our high pressure curves demonstrate clearly that one cannot estimate the fraction of hemoglobin inactivation from a pressure

range of only 0—1 atmosphere. When dealing with a Root effect there is always (in our material) a substantial rise beyond a pO_2 of 1 atmosphere. The pO_2 must usually be at least 10—20 atmospheres before the final plateau is reached (cp. Fig. 1 and SCHOLANDER and VAN DAM 1954, Figs. 3—4). The consequence of this may be seen from the following example. Root and IRVING (1941) published a series of careful analytical data on the effect of CO_2 on the oxygen dissociation curve of tautog. At 90 mm CO_2 pressure there was seemingly a clear Root effect with a plateau estimated at 55 % saturation, i. e., 45 % of the hemoglobin was inactivated. The highest oxygen tension used was a little less than 1 atmosphere. The data could be closely fitted to a hyperbola with an asymptote at 55 % saturation. Our tautog data (l. c., 1954, Fig. 3) show, however, that there is no such oxygen asymptote at this CO_2 pressure, but that the curve continues to rise and reaches some 90 % saturation at 40 atmospheres' oxygen pressure. For reasons stated above, a single hyperbola with an asymptote at 55 % saturation cannot be derived from the multiple component theory when dealing with 4 hemes. The found hyperbola must therefore be regarded as the sum of two curves, and one must postulate further that one or two more curves must be added onto these in order to account for the slow rise above 40 atmospheres. By suitable choice of constants this can probably be done, but such mathematical transactions can hardly disclose much of the underlying physical reality.

The slope from 0 to 1 atmosphere's pO_2 , both for this curve and for many other carefully determined curves showing a Root effect, is already as steep as possible, namely, corresponding to an n of one, and it is therefore impossible to describe in one equation the full extent of such a curve. It could possibly be approximated by choosing n as a small fraction of one, but this again would hardly have any physical meaning. In fact, when n is determined from our curves by plotting $\log HbO_2/Hb$ versus $\log pO_2$, it starts as a small fraction of one and increases to 2 or more as saturation is approached.

The data in Fig. 1, as well as our other high pressure observations, were obtained spectrophotometrically on whole blood and often under field conditions, and we cannot claim for them the high standard of accuracy applicable to much previous data obtained in the low pressure range by gasometric methods, but we believe the accuracy is good enough to illustrate the main points

we have made. In all events, it seems justified to say that present theories of oxygen dissociation do not apply well to fish blood. BLACK and IRVING (1938) and ROOT, IRVING and BLACK (1939) showed the rather disturbing fact that both the Bohr and the Root effects in the blood of some fishes are greatly diminished or abolished when the blood is hemolyzed, *i. e.*, one cannot from the behavior of a solution of fish hemoglobin predict the dissociation curve of whole blood. The difficulty arises that one does not know to what extent conditions in the plasma, such as acidity and other factors, reflect the conditions within the cells.

It may be concluded that the current theories, designed to explain the oxygen combination with hemoglobin in the relatively simple system of mammalian blood, present considerable difficulties when applied to fish blood, especially when the pressure range is extended beyond that of 1 atmosphere's p_{O_2} . These theories also seem unable to account for the action of acid and CO_2 on fish blood.

Summary.

The oxygen dissociation curve of the common silver hake (*Merluccius bilineatus*) is described at pH 5.9–7.7 and p_{O_2} 0.2–140 atmospheres. At pH 7 the curve has a conspicuous plateau before it reaches full saturation at about 100 atmospheres. It is pointed out that high pressure curves from fish in general cannot readily be described or interpreted in terms of current theories evolved for mammalian blood.

Acknowledgment.

The present data were obtained on a deep sea cruise conducted by Mr. W. C. SCHROEDER from the Woods Hole Oceanographic Institution. I am indebted to Dr. L. VAN DAM for help in securing the data, which I have had the privilege of discussing with Dr. A. C. REDFIELD and Dr. F. J. W. ROUGHTON.

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The Mechanism of the Disruption¹ of Mast Cells Produced by Compound 48/80.

By

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Compound 48/80, extracts from the eelworm of swine (*Ascaris lumbricoides*) and from the jellyfish (*Cyanea capillata*), liberate histamine from the perfused paw of the cat and cause disruption of mast cells in the rat mesentery (HÖGBERG, THUFVESSON and UVNÄS 1956 a). The liberation of histamine as well as the disruption of mast cells induced by these agents were observed to be blocked by a polysaccharide fraction isolated from hip seeds (HÖGBERG, SÜDOW, THON and UVNÄS, 1956 b).

It is known that high molecular weight polyanions produce a complete, reversible inhibition of different enzymes (DICZFALUSY, FERNÖ, FEX, HÖGBERG, LINDEROT and ROSENBERG 1953, FERNÖ, FEX, HÖGBERG, LINDEROT and ROSENBERG 1953). The inhibition caused by these compounds, is believed to be due to blocking of the "active centers", the free amino groups, of the enzyme.

In an earlier paper we hypothesized that the polysaccharides in the hip seed extracts produced inhibition of histamine liberation by interfering with some enzymic process involved in the liberation mechanism common to the liberators studied. In the

¹ We have used the term disruption to designate the discharge of mast cell granules, since the term has become widely accepted in the literature. The term must not be taken literally, for it is still uncertain whether the mast cell membrane is really destroyed and the granules "leak" out of the cell or if the granules are actively transported ("secreted") through a more or less intact cell membrane.

present investigation we have sought to find support for the assumption that 48/80 and some other positively charged histamine liberators, for instance protamine, cause disruption of mast cells by activating an enzymic mechanism.

Methods.

Technique for Observations on the Disruption of Mast Cells.

A slight modification of the technique described by NORTON (1954) was used. The rat was killed by a blow on its head and the abdomen immediately opened. Suitable pieces of the mesentery were removed and placed in solutions of the substances to be tested. These substances had been dissolved beforehand in 5 ml portions of Tyrode's solution. When the inhibitory action of metal ions was tested, the mast cells were incubated in physiological saline and the pH if necessary adjusted to about pH 7.4 by adding NaOH. Usually two pieces of mesentery were used in each concentration of drugs and one piece for control. Per cent disruption is in every experiment calculated after subtraction of the disruption observed in the control.

Materials.

Inhibitor from hip seeds.

The inhibitor was prepared from air-dried ripe hips, using the method described by HÖGBERG, SÜDOW, THON and UVNÄS (1956 b).

Lecithin.

Native lecithin of hen egg was purified according to HANAHAN (1954).

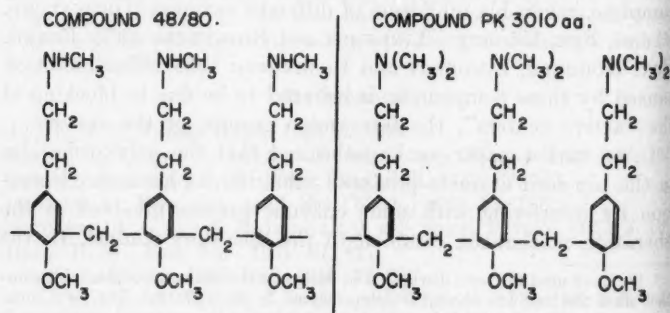


Fig. 1

Table 1.

The effect of various inhibitors on the disruption of mast cells produced by compound 48/80.

Inhibitor	Conc. of Inhibitor M	Conc. of 48/80 $\mu\text{g/ml}$	% Disruption	Inhibitor	Conc. of Inhibitor M	Conc. of 48/80 $\mu\text{g/ml}$	% Disruption
—	—	5	100	d,l-Histidine	6.10^{-4}	5	100
AlAc ₃	5.10^{-4}	5	100	"	6.10^{-3}	5	0
"	5.10^{-3}	5	0	Iodoacetic acid	5.10^{-4}	5	100
CuAc ₂	5.10^{-4}	5	100	"	5.10^{-3}	5	0
"	5.10^{-3}	5	0	Phenol	1.10^{-3}	5	100
H ₂ O ₂	5.10^{-4}	5	6	"	2.10^{-3}	5	15
"	5.10^{-3}	5	0	Phenylhydrazine	7.10^{-4}	5	0
KCN	1.10^{-3}	5	100	"	7.10^{-3}	5	0
"	2.10^{-4}	5	0	Phlorizin	2.10^{-4}	5	100
MgCl ₂	5.10^{-4}	5	100	"	2.10^{-3}	5	100
"	5.10^{-3}	5	100	α -Tocopheryl phosphate	1.10^{-3}	5	100
NaF	$2.5.10^{-3}$	5	100	"	1.10^{-4}	5	0
"	$2.5.10^{-2}$	5	100	"	"	"	"
PbAc ₂	$2.5.10^{-4}$	5	90	Tyramine	4.10^{-3}	5	100
"	$2.5.10^{-3}$	5	0	"	1.10^{-3}	5	0
ZnCl ₂	5.10^{-3}	5	100	Polyphloreitin phosphate	25 $\mu\text{g/ml}$	5	90
"	3.10^{-4}	5	0	"	50	5	10
Aniline	1.10^{-4}	5	100	"	100	5	0
"	1.10^{-3}	5	100	"	100	10	10
l-Cysteine	5.10^{-4}	5	100	"	"	"	"
"	5.10^{-3}	5	15	Polyestradiol phosphate	100	50	10
2,4-Dinitrophenol	5.10^{-4}	5	100	"	200	5	10
"	5.10^{-3}	5	0	"	400	5	0
"	"	"	"	"	800	5	0

β -Monostearoyllecithin.

As a lysolecithin β -monostearoyllecithin was prepared by the method of HANAHAN (1954).

1,3-Diphosphoimidazole.

A preparation of the calcium salt of diphosphoimidazole was kindly supplied by Dr. THOMAS ROSENBERG. For use, the calcium salt was converted to the potassium salt with Dowex 50 resin in K-form.

Polyphloreitin phosphate.

This inhibitor was prepared by the method of DICZFALUSY, FERNÖ FEX, HÖGBERG, LINDEROT and ROSENBERG (1953).

Compound 48/80 and Pk 3010 aa (Fig. 1).

The condensation product of p-methoxyphenethylmethylamine with formaldehyde (compound 48/80) was prepared according to the method

Table 2.

The effect of KCN on the disruption of mast cells produced by compound 48/80.

Concentration of Liberator $\mu\text{g/ml}$	Concentration of KCN M	% Disruption
5	4.10^{-3}	0
10	4.10^{-3}	0
20	4.10^{-3}	0
40	4.10^{-3}	0
100	4.10^{-3}	2

Table 3.

The effect of H_2O_2 on the disruption of mast cells produced by compound 48/80.

Concentration of Liberator $\mu\text{g/ml}$	Concentration of H_2O_2 M	% Disruption
5	1.10^{-3}	0
10	1.10^{-3}	40
20	1.10^{-3}	80
40	1.10^{-3}	100
100	1.10^{-3}	100

described by BALTZLY, BUCK, DE BEER and WEBB (1949)¹. For our experiments with diphosphoimidazol and other substances reacting with primary and secondary amines, we used, instead of 48/80, the corresponding tertiary amine as liberator (Pk 3010 aa). Both liberators are equally active when tested on rat mast cells.

Enzymes.

The lecithinase A² used in the present experiments was prepared from the venom of several species of snakes, *Crotalus atrox*, *Crotalus adamanteus*, *Crotalus terrificus* and *Naja Naja*³, by the method of SLOTTA and FRAENCKEL-CONRAT (1938), and from bee venom with ion exchange chromatography on Amberlite IRC-50 (XE-64) by the method of HÖGBERG (1957).

¹ We are indebted to Dr. H. FEX at the Leo Research Laboratories for preparing compounds 48/80 and Pk 3010 aa.

² Influenced by a recent review by HANAHAN (Progress in the Chemistry of Fats and Other Lipids, Vol. IV, London, 1957) we have preferred the name lecithinase before phospholipase.

³ Obtained from Ross Allen's Reptile Institute, Silver Springs, Florida.

Table 4.

The effect of various enzymes on the mast cell membrane.

Enzyme	Max. conc. used μ g or units/ml	% Disruption
Acetylcholinesterase	400	0
α and β amylases	500	0
Pectinesterase	500	0
Hyaluronidase	20 I. U.	0
β -Glucuronidase	1,680 Fishnam units	0
Hexokinase	500	0
Cozymase	400	0
Lipas (pancreas)	400	0
Lecithinase A (Bee venom)	200	100
Malic dehydrogenase	200 U.	0
Trypsin	2,500	10
Carboxypeptidase	400	0
Thrombin (1)	19 U.	2
Fibrinogen (2)	600	5
1 + 2		0
Plasminogen (3)	1 ml	0
1 + 2 + 3		20
Streptokinase (4)	1,000 U.	10
1 + 2 + 3 + 4		10
4 + rat serum	+ 0.4 ml	0
Pancreatin	1,000	0
Urease	500	0
Ribonuclease	1,000	0
Desoxyribonuclease	1,000	0
Uricase	500	0
Phosphatase, alkaline	500	10
Phosphodiesterase (snake venom)	500	0
ATP-ase	100	0
Cytochrome C	500	0
Carbonic anhydrase	500	0

No qualitative differences in the results were noted when using lecithinases from these different sources.

Hyaluronidase was prepared from bull testes by the method of HÖGBERG (1954) and contained about 54,000 I. U. per mg N.

Other materials were obtained from standard commercial sources. Glass redistilled water was used to make the reagents and to rinse glassware.

Results.

Effect of Enzyme Inhibitors.

The mast cells were incubated with 48/80 (5 μ g/ml) in the presence of several enzyme inhibitors. As is evident from table 1,

Table 5.

Disruption of mast cells with lecithinase A from different sources.

Lecithinase A from	Conc. µg/ml	% Disruption
Naja Naja	2.5	25
"	5	50
"	10	70
"	25	100
"	50	100
Crotalus atrox	2.5	0
"	5	60
"	10	100
"	20	95
"	40	95
"	80	100
Crotalus adamanteus ..	1	0
"	2	20
"	5	20
"	10	15
"	20	52
"	25	90
"	50	90
"	100	100
Crotalus terrificus ...	2	0
"	5	0
"	10	0
"	20	5
"	50	100
Bee venom	0.5	5
"	1	20
"	1.9	50
"	2.5	82
"	5	100

the disruption of mast cells was completely inhibited in the presence of some metal ions and some other typical inhibitors as phenol, phenylhydrazine and iodoacetic acid in concentrations around 10^{-3} M or lower. Especially α -tocopherylphosphate had a marked blocking action, and also the synthetic high molecular weight enzyme inhibitors polyphloretin phosphate and polyestradiol phosphate. Fluoride, phlorizin and aniline had no similar action, nor had adrenaline, noradrenaline, ATP, bufotenine, 5-hydroxytryptamine, tryptophane, which substances are not listed in the table.

Experiments with higher concentrations of 48/80 showed that the blocking action of some inhibitors was independent of the con-

Table 6.

Influence of inhibitors on the action of lecithinase A on mast cells.

Inhibitor	Conc. of Inhibitor M	Conc. of Lecithinase A $\mu\text{g/ml}$	% Disruption
		10	100
		20	100
		40	100
		80	100
CuAc ₂	5.10 ⁻⁴	10	10
"	5.10 ⁻³	10	0
MgCl ₂	5.10 ⁻⁴	10	100
"	4.10 ⁻³	10	100
NaF	5.10 ⁻⁴	10	100
"	4.10 ⁻³	10	100
PbAc ₂	5.10 ⁻⁴	10	0
"	4.10 ⁻³	10	0
ZnCl ₂	1.10 ⁻⁴	10	0
"	1.10 ⁻³	10	0
Phlorizin	5.10 ⁻⁴	20	100
"	4.10 ⁻³	20	100
Phenylhydrazine	1.10 ⁻³	20	0
"	5.10 ⁻³	20	0
α -Tocopheryl phosphate ..	1.10 ⁻⁴	20	0
"	4.10 ⁻³	20	0
Pectic acid fraction	1000 $\mu\text{g/ml}$	10	0
" "	100 "	10	0
" "	10 "	10	100
Polyphlorethin phosphate ..	100 "	10	0
" "	50 "	10	0
" "	25 "	10	50

centration of 48/80 at least up to concentrations 20 times higher than those normally used (Table 2).

In other cases, however, the degree of inhibition was dependent on the concentration of the liberator. Such inhibitors (*e. g.* oxidizing agents) probably attack and inactivate the liberator (Table 3).

Action of Various Enzymes on the Mast Cell Membrane.

The mast cells were incubated with several different enzymes.

Only one of all the enzymes investigated had the ability to disrupt the mast cell (Table 4). The active enzyme was a lecithinase A, isolated from bee and snake venoms. All the lecithinase preparations were highly potent in their disrupting activity (see

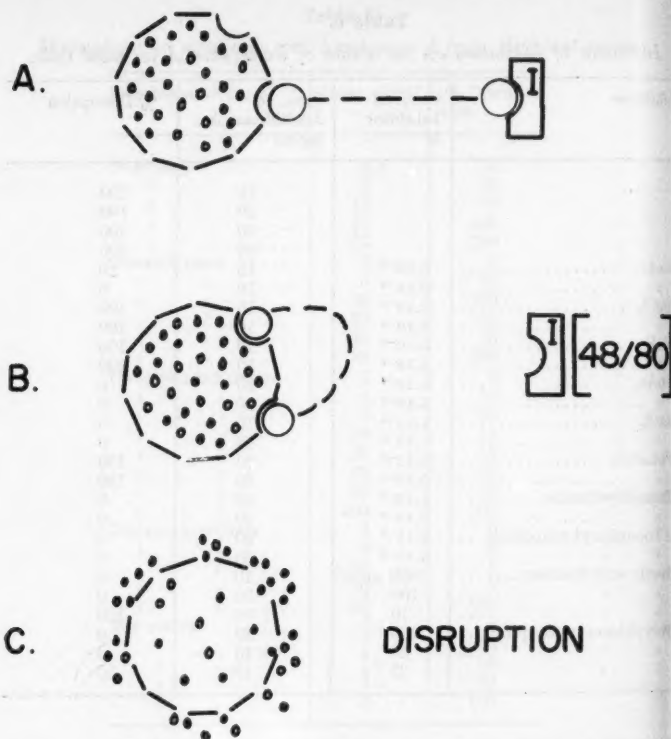


Fig. 2. Hypothesis to explain the fragmentation of mast cells produced by 48/80.

Table 5). It is also interesting to notice that lecithinase A is not inactivated by pancreatin, trypsin and carboxypeptidase.

Comments.

Of special interest to the later discussion is the fact that the enzymes trypsin, ribonuclease and fibrinolysin (activated by streptokinase or chloroform) and hyaluronidase, were unable to disrupt the mast cells even in high concentrations. The observation that the mast cells were disrupted only by lecithinase A suggested to us that a lytic enzyme might be activated by 48/80. The action of enzyme inhibitors on the disrupting effect of lecithinase A was therefore studied.

Table 7.

Inhibition of the action of 48/80 on mast cells at high lecithin concentration.

48/80 μg/ml	Lecithin μg/ml	% Disruption
5	—	100
5	50	100
5	125	100
5	250	80
5	500	10
5	1,000	0
5	1,125	0

Action of Enzyme Inhibitors on Lecithinase A.

The mast cells were incubated with lecithinase A prepared from bee venom, and the effect was observed of some of the inhibitors which blocked the action of 48/80 (Table 6).

Polyphloretin phosphate and the pectic acid fraction from hip seeds, which had previously been shown to block the disruption of mast cells caused by 48/80 (HÖGBERG et al., 1956 b), also blocked the disrupting action of lecithinase A (Table 6).

As is illustrated in tables 1 and 6, the inhibitory effects of the various enzyme inhibitors on the disrupting action of 48/80 and of lecithinase A ran surprisingly parallel. Note the strong inhibitory action of zinc ions, phenylhydrazine and α -tocopheryl phosphate.

Our observations as to the action of enzyme inhibitors on the disruption of mast cells produced by 48/80 and by lecithinase A accorded with our assumption that 48/80 exerts its effect by activating an enzyme which acts upon lecithin and related compounds. According to HUGHES (1935) the lecithinase A has two active amino groups. We therefore postulated that an enzyme having two positive sites was attached to the mast cell, one of them being anchored to its membrane (A in fig. 2). The other was thought to be blocked with a negatively charged inhibitor. — We further assumed that 48/80 interacts with the inhibitor (B in fig. 2), whereby the enzyme becomes active and the mast cell membrane disrupts (C in fig. 2). If the enzyme contains two spatially independent points of interaction with the substrate, the lecithin membrane, the presence of substrate in excess should lead to the formation of inactive enzyme. Assuming this to be correct, the

Table 8.

Influence of inhibitor on the action of lysolecithin on mast cells.

Lysolecithin μg/ml	Hip seeds comp. μg/ml	% Disrup- tion
—	—	0
25	—	0
50	—	0
100	—	30
200	—	63
500	—	100
1,000	—	100
500	1,000	100
500	500	100
500	100	100

enzyme activity ought to be inhibited by lecithin in excess; in other words lecithin should inhibit the disruptive effect of 48/80. Table 7 shows that high concentrations of lecithin — above 500 μg/ml — inhibit the rupture produced by 48/80.

From their work on the histamine release caused by cobra venom in the perfused guinea-pig lung FELDBERG and KELLAWAY (1938), suggested that a lytic substance, *e. g.* lysolecithin, was formed and acted as a histamine releasing agent. From table 8 it is evident that lysolecithin (β -monostearoyllecithin) is able to disrupt mast cells, although not until it reaches very high concentrations. When compared with the action of *e. g.* 48/80, it is a very weak disrupting agent. Furthermore, its disrupting action is not blocked by the hip-seed inhibitor, even in comparatively high concentrations (Table 8). For the action of the hip-seed inhibitor on the effect of 48/80 see HÖGBERG et al. (1956 b).

Action of Temperature.

The mast cells were incubated at various temperatures, from 0° C up to 60° C and the effect of 48/80 and of lecithinase A was observed. At 0° C no rupture was seen, either with 48/80 or with lecithinase. With increasing temperature the disrupting action of both agents appeared. The effect of 48/80 was blocked at 45–50° C and above, while the effect of lecithinase A was influenced only in low concentrations by the high temperatures of 50 and 60° C and above (see table 9).

Table 9.

Influence of temperature on the action of 48/80 and lecithinase A on mast cells.

Histamine Liberator	Conc. of Histamine Liberator	% Disruption							
		0° C	10° C	20° C	30° C	37° C	40° C	50° C	60° C
48/80	20	0	50	100	100	100	100	5	0
"	10	0	60	100	100	100	100	10	0
"	5	0	30	100	100	100	100	0	3
"	2.5	0	0	100	100	100	100	0	0
"	1.25	0	0	95	95	100	100	0	0
"	0.5	0	0	10	18	100	90	0	0
"	0.25	0	0	0	0	20	15	0	0
"	—	0	0	0	0	0	0	3	0
Lecithinase	50	0	—	—	100	100	100	100	100
"	20	0	—	—	100	100	100	100	100
"	10	0	—	—	100	100	100	100	100
"	5	0	—	—	100	100	100	55	100
"	2.5	0	—	—	75	80	100	6	60
"	1	0	0	0	0	60	70	40	30
"	—	0	0	0	0	0	0	0	0

The observation that the action of 48/80 is inactivated by temperatures between 40 and 50° C accords with the assumption that an enzymic process is involved in the disrupting action of 48/80. The inhibitory effect of temperatures between 40 and 50° C on the action of 48/80 is *irreversible*. Mast cells heated for 10 min. at 60° C are resistant to subsequent incubation with 48/80 at 37° C, but the membrane is still vulnerable to the action of lecithinase A and of surface-active agents such as lysolecithin and octylamine. Incubation with lecithinase A and with 48/80 at 0° C does not inhibit the disruption of mast cells at subsequent incubation with those agents at 37° C.

Comments.

The fact that the lecithinase A isolated from bee venom is not inactivated by heating to 60° C, while the postulated cell membrane enzyme is inactivated at that temperature, cannot be explained by our experiments; but enzymes are often observed to change character in various respects when isolated, the degree of purity and the source being of great importance for the chemical properties of the enzyme preparation.

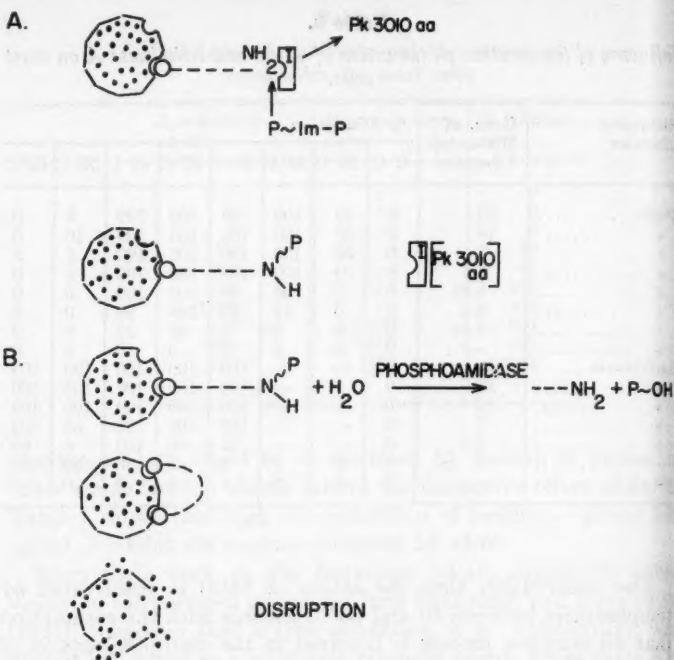


Fig. 3. A tentative explanation of the blocking action of "phosphorylation" of mast cells with diphosphoimidazole (A), and the subsequent fragmentation by phosphoamidase (B). For explanation see text p. 357.

Reversible and Irreversible Inactivation of Active Groups in "the Mast Cell Membrane Enzyme".

According to our working hypothesis, 48/80 and other positively charged polyvalent liberators, as protamine, activate an enzyme outside the cell which acts upon the membrane. In the following study, the essentiality of amino groups of this enzyme was investigated. As carriers of a positive charge, these groups could be expected to play a role in the electrostatic interaction of enzyme and inhibitor.

The mast cells were simultaneously incubated with NH_2 reacting agents and a liberator. As liberator we used, instead of 48/80, the corresponding tertiary amine (Pk 3010 aa; see fig. 1).

Recently, RATHLEV and ROSENBERG (1956) described a new

Table 10.

The inhibitory effect of diphosphoimidazole on mast cell fragmentation by Pk 3010 aa and lecithinase A.

No.	Pk 3010 aa μg/ml	1107/II μg/ml	Incuba- tion 20 min.	Washing	Lecithin- ase A μg/ml	Incuba- tion 20 min.	Disrup- tion of mast cells (%)
1	—	—	×	×	—	×	0
2	—	—	×	×	—	×	3
3	—	—	×	×	20	×	100
4	—	—	×	×	20	×	100
5	5	100	×	×	—	×	2
6	5	100	×	×	—	×	15
7	5	100	×	×	20	×	100
8	5	100	×	×	20	×	75
9	5	—	×	×	—	×	100
10	5	—	×	×	—	×	100

Result: Phosphorylation with diphosphoimidazole blocks the fragmentation produced by Pk 3010 aa. Note that lecithinase A still disrupts the cells. Pk 3010 aa = histamine liberator with $N(CH_3)_2$ groups. 1107/II = diphosphoimidazole.

method for phosphorylation of amines in aqueous neutral solution with a new compound, 1,3-diphosphoimidazole (DPI). Primary aliphatic amines, especially, seem to be easily phosphorylated by DPI.

One of us (B. H.) has found that DPI inactivates some enzymes with essential amino groups, namely, hyaluronidase and lecithinase A. In contrast to the relatively slow reaction of DPI with the amines described by RATHLEV and ROSENBERG, DPI has been found to cause an instantaneous inactivation of these enzymes.

Incubation of mast cells simultaneously with DPI and the histamine liberator Pk 3010 aa inhibits the disruption mechanism completely. As shown in tables 10 and 11, 5 μg/ml Pk 3010 aa produces a 100 per cent disruption of the mast cells. If 100 μg/ml DPI is added to the bath, the action of Pk 3010 aa is blocked. In our opinion the explanation is as follows (see Fig. 3 A).

Pk 3010 aa causes — as does 48/80 — disruption of the mast cells by removal from the mast cell enzyme of a normally occurring inhibitor. This removal of the inhibitor activates the enzyme situated at the membrane, and the latter is disrupted. However, in the presence of DPI the essential NH_2 groups of the enzyme become phosphorylated as soon as the inhibitor is removed by Pk 3010 aa. Thereby, the enzyme is inactivated and no destruction

Table 11.

The inhibitory effect of diphosphoimidazole on mast cell fragmentation by Pk 3010 aa and 48/80.

No.	Pk 3010 aa $\mu\text{g/ml}$	1107/II $\mu\text{g/ml}$	Incuba- tion 20 min.	Washing	48/80 $\mu\text{g/ml}$	Incuba- tion 20 min	Disruption of mast cells (%)
1	—	—	×	×	—	×	30
2	—	—	×	×	—	×	0
3	—	—	×	×	5	×	100
4	—	—	×	×	5	×	100
5	5	100	×	×	—	×	0
6	5	100	×	×	—	×	5
7	5	100	×	×	5	×	15
8	5	100	×	×	5	×	10
9	5	—	×	×	—	×	100
10	5	—	×	×	—	×	100

Result: Phosphorylation with diphosphoimidazole blocks the fragmentation produced by Pk 3010 aa and 48/80. Pk 3010 aa = histamine liberator with $\text{N}(\text{CH}_3)_2$ groups. 1107/II = diphosphoimidazole.

of the cell membrane occurs (nos. 5 and 6 in Table 10 and 11). The fact that the cell membrane remains sensitive to the action of lecithinase after treatment with DPI will be seen from nos. 7 and 8 in table 10, where 20 $\mu\text{g/ml}$ of lecithinase was added after washing out the excess of DPI. The action of 48/80 added to these

Table 12.

Fragmentation of "phosphorylated" mast cells after treatment with N-P splitting enzyme (phosphoamidase).

No.	Pk 3010 aa $\mu\text{g/ml}$	1107/II $\mu\text{g/ml}$	Incuba- tion 20 min.	Washing	Phospho- amidase $\mu\text{g/ml}$	Incuba- tion 20 min.	Disruption of mast cells (%)
1	—	—	×	×	—	×	0
2	—	—	×	×	100	×	3
3	5	—	×	×	—	×	100
4	5	—	×	×	—	×	100
5	5	100	×	×	—	×	0
6	5	100	×	×	—	×	5
7	5	100	×	×	100	×	100
8	5	100	×	×	100	×	78

Result: The blocking produced by phosphorylation of mast cell enzyme with diphosphoimidazole is abolished by dephosphorylation with phosphoamidase. Pk 3010 aa = histamine liberator with $\text{N}(\text{CH}_3)_2$ groups. 1107/II = diphosphoimidazole.

Table 13.

*Influence of antivenin on the action of 48/80 and lecithinase A
(Crotalus atrox) on mast cells.*

Conc. of 48/80 $\mu\text{g/ml}$	Conc. of lecithinase A $\mu\text{g/ml}$	Conc. of Antivenin in $\mu\text{g/ml}$	% Disrup- tion
5	—	—	100
5	—	10,000	0
5	—	5,000	0
5	—	2,500	0
5	—	1,000	65
—	50	—	100
—	50	10,000	0
—	50	5,000	0
—	50	2,500	10
—	50	1,250	45
—	50	1,000	75
—	50	500	100

washed mast cells was still blocked, due to the previous phosphorylation (nos. 7 and 8 in Table 11).

Now, if essential NH_2 groups in "the mast cell enzyme" were phosphorylated, a N—P splitting enzyme should reactivate the enzyme and cause disruption (see fig. 3 B). Table 12 illustrates experiments with mast cells inhibited by DPI. These cells are stable for 48/80 but *e. g.* a phosphoamidase can act as a liberator.

Acetylation of the mast cell enzyme was accomplished by incubation of the rat mesentery fragments for 10 minutes at 37°C in 5 ml Tyrode solution containing 0.01 ml acetic anhydride and $20\text{ }\mu\text{g/ml}$ Pk 3010 aa. The mesentery fragments were then washed in 0.9 % NaCl solution and incubated with 48/80 ($20\text{ }\mu\text{g/ml}$) for 10 minutes. No mast cell disruption was observed.

According to NYGAARD (1956) the acetyl-amino linkage is stable in M glycine at pH 9.5 for 5 minutes, but phenol acetate and thiol acetate are not.

No activation of the enzyme took place after incubation in M glycine at pH 9.5 for 5 minutes. Therefore, neither phenyl acetate nor thiol acetate appear to have formed with acetylation, thus indicating that the acetylation was specific for amino groups in the enzyme. It was interesting to observe that in the controls disruption appeared on incubation in M glycine at pH 9.5 indicating, according to our theory, a dissociation of enzyme-true inhibitor.

Table 14.

Influence of immune serum produced against lecithinase A (bee venom) on the action of 48/80 and lecithinase A.

Liberator	Conc. of Liberator $\mu\text{g/ml}$	Immune Serum (rabbits) ml	Normal Serum (rabbits) ml	% Disruption
48/80	5	—	—	100
"	5	—	2.0	100
"	5	—	1.0	100
"	5	2.0	—	0
"	5	1.0	—	7
Lecithinase A	20	—	—	100
"	20	—	2.0	100
"	20	—	1.0	100
"	20	2.0	—	0
"	20	1.0	—	0

Experiments with formaldehyde (10^{-3} M), phenylisocyanate ($0.1 \mu\text{l}$), benzoylchloride ($0.1 \mu\text{l}$) and 5-dimethylamino-1-naphthalene sulfonyl chloride ($20 \mu\text{g}$) (according to WEBER 1952) showed that the enzyme is inhibited by these amino group reagents.

Similar results were obtained with lecithinase A. Incubation with the above-mentioned phosphorylating and acetylating reagents inhibited the ability of the enzyme to disrupt the mast cells. The inhibition caused by phosphorylation with DPI could be abolished by incubation with phosphoamidase. The inhibition caused by acetylating with acetic anhydride was not reserved by incubation in M glycine.

Influence of Immune Serum on the Action of 48/80 and lecithinase on Mast Cells.

It has previously been shown (BELFENTI 1934, FRANCIOLI 1938) that homologous antisera inhibit the action of snake venom lecithinase A.

We therefore studied the effect of polyvalent antivenin (immune serum produced against snake venoms) on the action of 48/80 and lecithinase A on mast cells. The antiserum used was "Antivenin polyvalent" manufactured by Wyeth Inc. of Philadelphia. Table 13 shows that large doses of antivenin produced inhibition of both lecithinase A and 48/80.

To produce specific sera against our lecithinase A, rabbits were immunized with lecithinase A from bee venom. In a typical pro-

cedure the animal was given one intravenous injection daily for 7 days and allowed to rest for 3 days. To avoid the danger of anaphylactic shock, in the following 3 weeks the antigen preparation was given intramuscularly. About 150 mg of antigen per 3 kg rabbit, divided into about 20 injections, was the usual dosage. After immunization, the antisera were screened for their inhibitory effect on the action of 48/80 and lecithinase A on mast cells (Table 14). Immune serum produced against lecithinase A from bee venom, inhibited in high doses the disruption of mast cells caused by 48/80 and lecithinase A.

Discussion.

In our experiments we have made some observations indicating that compound 48/80 disrupts mast cells with the mediation of an enzymic mechanism.

The effect of 48/80 can be blocked by several enzymic inhibitors and is dependent on the temperature. Below 20° C and above 45–50° C the disruptive action of 48/80 is blocked. The block produced by temperatures above 45°–50° C is irreversible.

Of several enzymes investigated with respect to their ability to disrupt mast cells only one, lecithinase A, was found to possess that ability. Lecithinase A was isolated from bee venom and several snake venoms. The disruptive action of lecithinase A on the mast cell membrane was observed to be inhibited by the same agents that inhibited the action of 48/80. There was a rather good quantitative correlation between the inhibitory effects on the action of 48/80 and of lecithinase A. Worth noting are, for instance, the pronounced inhibitory effects of zinc ions and of *α*-tocopheryl phosphate.

The fact that lecithin in excess inhibits the disruption caused by 48/80 supports the theory that the mast cell enzyme acts on phospholipides. Further, the mast cell enzyme seems to contain — as does lecithinase A — essential amino groups susceptible to NH_2 reagents. Phosphorylation and acetylation of the NH_2 groups inactivates lecithinase A. Similarly, incubation of mast cells with phosphorylating or acetylating agents renders these cells stable to 48/80 and other negatively charged liberators such as protamine. The cells remain sensitive to lecithinase A and various lipid dispersing agents (lysolecithin, octylamine, etc.).

The observations presented above agree with our working hypothesis that a lytic enzyme is localized to the mast cell membrane. The enzyme has amino groups essential for its activity. It is attached to the phospholipid part of the cell membrane; the other is blocked by an inhibitor in ionic linkage to the amino group. The removal of the inhibitor by a negatively charged histamine liberator such as 48/80 or protamine activates the enzyme and the mast cell disrupts. Removal of the inhibitor by a histamine liberator such as the tertiary amine Pk 3010 aa in the presence of a phosphorylating (or an acetylating) agent, does not cause disruption of the mast cells but produces instead mast cells resistant to the subsequent action of 48/80. The P—N bond (or the acetyl linkage) prevents the mast cell enzyme from breaking down its substrate (the cell membrane). If the P—N bond were broken, the enzymes should become active and the cells disrupt. The fact that phosphoramidase causes a disruption of phosphorylated mast cells closely accords, therefore, with our hypothesis.

How does our hypothesis fit the present conception of the histamine liberation?

Recent years have brought cogent evidence that most of the releasable histamine in the tissues is localized to the mast cells. The release of histamine by 48/80 and other polymer substances is due to the emptying of the histamine content from these cells. The cells are observed to disrupt or at least to lose their basophilic granulation during this process.

The mast cell histamine is probably localized to basophilic granules, and most evidence indicates that it is there only in loose linkage to other compounds and remains in the granules, since the latter are surrounded by a membrane through which it cannot normally pass (McINTIRE, ROTH and SPROULL 1950, McINTIRE 1956, TRETHEWIE 1938, MOTTA, BERALDO, FERRY and JUNQUEIRA 1954, BLASCHKO, HAGEN and WELCH 1955 etc.).

The histamine-containing subcellular elements in the mast cells are rather fragile and their isolation requires special arrangements to prevent their disintegration and the escape of histamine. It is therefore possible that breaking down of the mast cell membrane, or rather changes in its permeability, will cause physicochemical disturbances inside the cell sufficient to produce a leakage of histamine into the cell sap and then out of the cell.

If the histamine is localized to the mast cell as described above,

a change in the mast cell membrane permeability should be one of the requirements of a theory explaining the release of histamine from the mast cell.

According to our hypothesis, 48/80 produces the required changes in the permeability of the mast cell membrane by activating a lytic enzyme localized on the cell membrane.

The intimate nature of the additional links in the chain of events which is started by 48/80 is obscure and, so far, can only be a matter of speculation. One possibility is, of course, that 48/80 triggers not only one but several enzymic mechanisms ultimately resulting in the discharge of histamine. JUNQUEIRA and BEIGUELMAN (1955) have reported, for instance, that low temperature, acid pH and several metabolic inhibitors prevent granule extrusion from rat mast cells observable after the addition of compound 48/80. Some of the inhibitors belonged to the SH blocking compounds, and their inhibitory action was believed to be on metabolic processes involved in the discharge of the basophilic granules. In this case, the transport of the histamine-carrying granules to the outside of the mast cell would not be passive but the result of active processes, "a secretion". In our view their results do not warrant such a conclusion since the concentrations of the inhibitors used by them are too high to yield specific inhibitory effects.

The question arises as to the extent to which a lytic enzyme may be assumed to take part in the mast cell disruption and the histamine liberation produced by other agents than 48/80. Our observations that histamine liberation caused by 48/80 as well as by the active fractions isolated from *Ascaris* and *Cyanea* is blocked by the same polysaccharide fraction from hip seeds, might indicate that 48/80 and at least some biologically occurring histamine-liberating agents activate a common link in the chain of processes which ultimately lead to the release of histamine. The common link might be the lytic enzyme localized to the mast cell surface.

As pointed out by MONGAR (1956), the activity of histamine releasers varies considerably depending on the species, the tissue and the method used for determining the activity. The discrepancy may be a thousandfold. For instance, the concentrations of 48/80 required for the release of histamine from perfused (FELDBERG and MONGAR 1954) or chopped (MONGAR and SCHILD 1952) guinea-pig tissue or from isolated intracellular granules from dog

liver (MACINTOSH 1956) are rather high and of about the same magnitude as those required for corresponding histamine release produced by simple amines such as octylamine. Under such conditions the histamine-releasing action of 48/80 might be attributed, we assume, to a direct lytic action on the extra- and intracellular membranes rather than to the activation of specific enzyme systems.

Mast cells in the guinea-pig lung have been observed to undergo disruption or to lose their basophilic granulation in anaphylactic reactions. It has been found, by us, that the histamine release from sensitized guinea-pig lung tissue which is caused by the addition of the antigen (horse serum or ovalbumin) can be inhibited by prior addition of the same polysaccharide fraction from hip seeds that was reported to block the mast cell disruption in the rat mesentery and the release of histamine from the perfused cat's paw, produced by 48/80 and the active agents obtained from *Ascaris* and *Cyanea* (HÖGBERG, SÜDOW, THON and UVNÄS 1956 b). We are therefore inclined to believe that the release of histamine from mast cells, occurring in anaphylactic reactions in guinea-pig lungs, involves the action of a lytic enzyme which is blocked by our polysaccharide fraction.

In anaphylactic shock there occurs, besides histamine, a "slow reacting substance" (SRS) with smooth muscle stimulating properties (KELLAWAY and TRETHEWIE 1940, BROCKLEHURST and MONGAR 1956). A factor having a similar smooth muscle stimulating action also appears after intravenous administration of large doses of 48/80 to dogs and cats. The chemical nature of the SRS occurring under the conditions mentioned above is unknown.

Some fatty acids have been shown to possess a high toxicity (WRETLIND 1957). It is also of interest that VOGT (1956) isolated a SRS from egg yolk incubated with lecithinase A. This SRS was identified as an unsaturated fatty acid. Another substance producing a slow contraction of guinea-pig gut *in vitro* was isolated from human plasma by GABR (1956) and found probably to be an unsaturated fatty acid.

Lecithinases are widely distributed enzymes in the body. If, therefore, the slow reacting substance occurring in anaphylactic reactions were shown to be a fatty acid or a phospholipid rest, it should support the hypothesis that a lecithinase is involved in the histamine liberating processes, especially if it could be

shown that the inhibition of the histamine release also prevented the occurrence of the SRS.

Preliminary observations at our laboratories have shown that the SRS which occurs in sensitized guinea-pig lung tissue on addition of the antigen (ovalbumin) is soluble in 80 % alcohol. When, after evaporation of the alcohol, the dried residue was shaken with ether to which a few drops of N HCl were added the active agent dissolved into the ether. If the ether was then shaken against alkaline water the SRS moved into the water. In other words, the SRS fraction behaved as a lipid soluble acid. Other observations have revealed that when the histamine release in the sensitized guinea-pig lung is blocked by polysaccharides, the SRS does not appear in the perfusate. These preliminary observations seem to support our lecithinase theory.

One of the arguments against a lecithinase theory could be that little or no hemolysis occurs in anaphylactic shock, in pepton shock, in intoxication with *Ascaris* products, etc. The absence of hemolysis is not, in our opinion, a valid objection to a lecithinase theory. If a lytic enzyme is anchored to the mast cell membrane, activation of the enzyme will not necessarily bring about a spread of active enzyme in the blood. An enzyme attached to the mast cell membrane or to membrane fragments is unlikely on any major scale to come into contact in active form with circulating blood corpuscles.

FELDBERG and KELLAWAY (1937) reported that on perfusion of guinea-pig lung with diluted solutions of three different snake venoms, each of the latter caused release of histamine. Many of the physiological effects of these venoms injected into dogs and cats were accordingly attributed to the effect of released histamine. In a later paper the same authors (FELDBERG and KELLAWAY 1938) ascribed the histamine-liberating capacity of the venoms to a lecithin-splitting enzyme which produced a lytic substance, lysolecithin. By its lytic action, lysolecithin should liberate histamine from its linkage with lipoproteins in the tissues.

The theory of FELDBERG and KELLAWAY was propounded at a time when the localization of histamine to the mast cells was unknown. Today the experimental evidence does not favor the view that histamine is linked to lipoproteins in the mast cells, at least not with bonds which require an enzymatic mechanism for their breakdown. It is true, however, that lysolecithin is able to disrupt mast cells, and the possibility cannot be excluded that

lysolecithin could act as mediator of a mast cell lecithinase. The observation that *in vitro* high concentrations of lysolecithin are required to cause a disruption of rat mast cells is not a cogent argument against the participation of lysolecithin, since the latter might well reach active concentrations at the cell membrane when the mast cell lecithinase becomes active and splits the membrane lecithin. On the other hand, the fact that lysolecithin has a weak lytic action *in vitro* does not necessarily mean, we would emphasize, that it plays a part in the breakdown of the mast cell membrane. The question whether or not lysolecithin mediates the lytic action of lecithinases is still open.

Finally, how does our lecithinase theory fit with the enzyme theories proposed by ROCHA E SILVA (1938), UNGAR (1947) and others, which postulate that histamine release is due to the activation of proteases? The simplest answer to this question is that our observations do not seem to support the protease theories. Trypsin and fibrinolysin and other proteases used by us were unable, even in high concentration, to disrupt mast cells *in vitro*. The argument that proteases of various types appear in anaphylactic reactions does not, in our opinion, lend much weight to the view that the histamine release is due to the activity of such enzymes. Leukocytes and thrombocytes contain activators of various proteases. Since immense numbers of those cells are destroyed in anaphylactic shock, it is not surprising that several proteases in the blood are activated — processes which are not necessarily connected with the histamine release. As pointed out by many authors, there is no convincing evidence that histamine occurs in the mast cells firmly linked to proteins. Hence, if proteases do not disrupt the mast cell membrane or change its permeability, then protease theories would seem to lack their *raison d'être*.

Space does not permit a discussion of all the numerous facets of histamine release problems. We would stress our view that if 48/80 disrupts the mast cells in the rat mesentery by activating a lytic membrane enzyme, then similar mechanisms probably take part under other conditions where histamine is released from mast cells. However, circumspection is required and evidence must be presented in each case, since different releasers, as already pointed out, may act by different mechanisms, and in fact there are already observations which show this to be the case.

Our observations spotlight a number of important questions. To what extent, for instance, can the stability of extra- and intra-

cellular membranes be attributed to the presence of enzymic inhibitors?

To what extent, too, can the release of humoral mediators at nerve terminals and in synaptic junctions, secretory processes the action of various drugs, and other events where changes in membrane permeability occur be explained by enzymic activation due to removal of inhibitors?

Summary.

The present studies were made on mast cells in the rat mesentery.

The disruption of mast cells by 48/80 was inhibited by various metal salts and other enzyme inhibitors.

Among numerous enzymes studied, only lecithinase A had the ability to disrupt mast cells. Trypsin, fibrinolysin, ribonuclease, hyaluronidase, etc. were inactive.

The disruptive action of lecithinase A was inhibited by the same agents which inhibited the action of 48/80.

The action of 48/80 was temperature-dependent. It was inhibited below 20° C and above 45°–50° C. The inhibition above 45°–50° C was irreversible.

The effect of 48/80 was inhibited by incubation with acetic anhydride and 1,3-diphosphoimidazole. The acetylation was not reserved under conditions which hydrolyzed phenyl acetate and thiol acetate. Dephosphorylation caused a disruption without the addition of a liberator.

Polyvalent immune serum against snake venom, "Antivenin", and specific immune serum (from rabbits) against lecithinase A (from bee venom), blocked in high doses the disruptive action of both lecithinase A and of 48/80.

The observations are considered to support the hypothesis that 48/80 acts by activating a lytic enzyme attached to the mast cell membrane. The activity of the enzyme is normally blocked by an inhibitor. When this inhibitor is removed, as by liberators such as 48/80, the enzyme becomes active and the membrane structures are attacked.

The implications of our mast cell enzyme theory for the histamine release produced by other releasers, for that occurring in ana-

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phylactic reactions, etc., are discussed. Attention is drawn to the possible applicability of similar enzyme theories to biologically occurring and pharmacologically produced changes in membrane permeability.

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The Effect of Tetraethylammonium (TEA) and Temperature on the Neuromuscular Block Produced by Magnesium.

By

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It has been known for many years that magnesium blocks neuromuscular transmission selectively in concentrations which do not affect the nerve or the muscle (review by ENGBAEK 1952). In recent years it has been established that by far the most important factor in the production of this blockage is an interference with the release of the physiological transmitter substance from the presynaptic nerve terminals. This was found by CASTILLO and ENGBAEK (1954) who analysed the electrical events at the myoneural junction during the action of magnesium. The inhibition of the release of acetylcholine has been confirmed by a more direct method of assaying the acetylcholine output from the perfused sympathetic ganglion (HUTTER and KOSTIAL 1954).

The depressant effect of magnesium at the myoneural junction is to a large extent antagonized by an excess of calcium. CASTILLO and ENGBAEK (1954) found that calcium effected an increased output of acetylcholine when this had been inhibited by magnesium, and thus relieved the neuromuscular block. HUTTER and KOSTIAL (1954) showed that calcium relieved the magnesium block by restoring the output of acetylcholine from the perfused sympathetic ganglion. Withdrawal of calcium, on the other hand, acted like addition of magnesium and produced junctional block

by lowering the acetylcholine output (CASTILLO and STARK 1952).

In a previous paper (STOVNER 1957 b) it has been shown that TEA can partly restore neuromuscular transmission depressed by lack of calcium. It is therefore possible that TEA, like calcium, opposes the neuromuscular block produced by magnesium. To the author's knowledge no reports have appeared in the literature on the effect of TEA on neuromuscular transmission depressed by magnesium.

The neuromuscular block due to lack of calcium was found to be dependent on temperature (STOVNER 1957 b). As this blockage is similar in its mechanism to that produced by excess of magnesium, it will be of interest to see if temperature influences the magnesium block in the same way.

The present work is an investigation of the action of TEA on the neuromuscular block produced by excess of magnesium. The investigation was carried out both on nerve-muscle preparations *in situ* in cats and rabbits, and on isolated diaphragm preparations from rats. Chemical sensitivity to acetylcholine and electrical records of end plate potentials were also studied in the rat diaphragm preparation during the interaction of TEA and magnesium. In addition the effect of lowering the temperature on the magnesium-blocked rat diaphragm preparation has been investigated.

Methods.

1. Experiments with isolated phrenic nerve diaphragm preparations from rats.

a) Contractions evoked by nerve stimulation.

The method of BÜLBRING (1946) was used with the modifications described in earlier papers (STOVNER 1957 a and b).

b) Contractions evoked by intravascular injection of acetylcholine.

The method of BURGEN et al. (1949) was used with the modifications described previously (STOVNER 1957 a).

c) End plate potentials.

The rat diaphragm preparation was set up for recording end plate potentials in the same way as before (STOVNER 1957 b). When neuromuscular transmission was depressed by magnesium, the end plate potentials were localized by moving the recording electrode. The low voltage monophasic non-propagated potential confined to a strip midway between the costal margin and the central tendon was regarded as the end plate potential.

2. Experiments with rabbits.

The experiments were performed on the flexor digitorum longus muscle of the rabbits' hind limb. The preparatory operation was done under ether anaesthesia supplemented by local anaesthesia. A tracheal cannula was inserted in the neck and positive pressure ventilation given with a mechanical device during administration of magnesium. The stimulating electrodes were placed on the sciatic nerve (NAESS 1950). The peripheral part of the severed sciatic nerve was stimulated supramaximally with rectangular impulses of 0.2 msec duration, and varying frequencies. The muscle was loaded with 200 g and isotonic contractions of the flexor digitorum longus muscle were recorded. Blood pressure was recorded continuously by means of a glass capsule manometer (ANDERSON 1941) and exact calibration done at the end of the experiment.

Approximately one and a half hours elapsed between the end of the operation and the beginning of the experiment, so only an insignificant amount of ether would be left in the blood. Barbiturates were avoided, as preliminary experiments had shown that even small amounts of these interfered with the action of TEA. During the experiments no anaesthesia was used apart from the magnesium. To obtain a constant neuromuscular block, a solution of $MgCl_2$ was infused continuously by a motor-driven syringe through a cannula in the jugular vein. While the sciatic nerve was stimulated and the contractions were being registered, the infusion speed was regulated until a constant neuromuscular block was achieved. When the neuromuscular block was constant TEA was injected through a cannula into the opposite jugular vein.

3. Experiments with cats.

The procedure was essentially the same as for the rabbit experiments.

Drugs and solutions employed.

Magnesium chloride (MERCK), calcium chloride (MERCK). The solutions of magnesium and calcium chloride were standardized by titrating the chloride by the Volhard method. Magnesium was added in exchange for an osmotically equivalent amount of sodium in the Tyrode solution. Tetraethylammonium (TEA) bromide (Boots), choline chloride (HOFFMANN-LA ROCHE), β -hydroxy-ethyl-triethylammonium chloride. This substance was synthesized at the Chemical Institute, University of Oslo and was obtained in crystalline form. Found by analysis: Cl: 19.8 %, N: 7.5 %. (Calculated: Cl: 19.55 %, N: 7.7 %.)

Results.

A. Neuromuscular block produced by magnesium on the isolated diaphragm of rats.

a) Effect of temperature.

The unaffected rat diaphragm preparations responded well

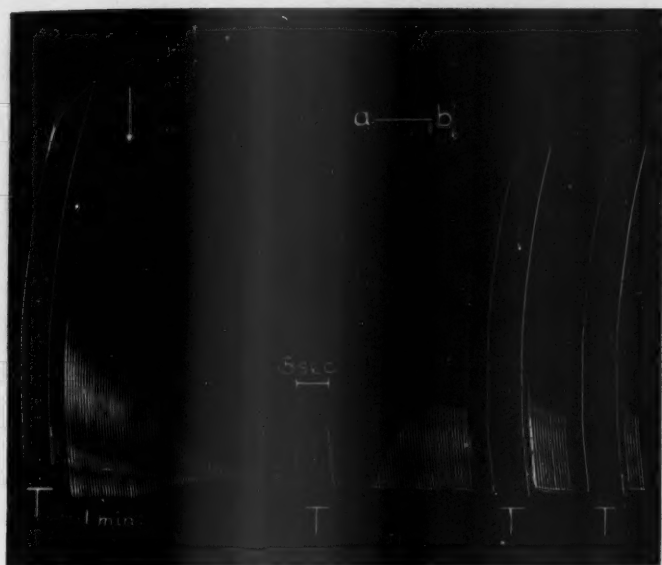


Fig. 1. Rat diaphragm. Neuromuscular block with magnesium. Restoring effect of low temperature. Arrow, change to bath fluid containing magnesium in a concentration of 6 mM. a—b, lowering of temperature from 37° C to 22° C. T, tetanic stimulation (50 per sec) for 5 sec. Between the periods of tetanic stimulation, single supramaximal nerve stimulation (6 per min). Rapid kymograph (1.5 mm per sec) during tetanic stimulation. Time in minutes.

both to single and tetanic nerve stimulation between 37° C and 22° C. On cooling from 37° C the responses to single nerve stimuli were increased somewhat and reached a maximum between 30° C and 25° C, where the contraction height was from 10 to 30 % higher than at 37° C. Below 25° C the twitch decreased slowly and was only slightly higher at 22° C than at 37° C. The speed of contraction, however, was considerably reduced. No significant change in the tetanic responses occurred.

At 37° C the responses to single nerve stimulation (6 per min) were just abolished with a concentration of about 6 mM magnesium in the bath fluid. The muscle still responded to tetanic nerve stimulation (50 per sec) although the height of the tetanic responses was markedly reduced. When the temperature of the bath was lowered to 22° C an almost complete restoration of the responses to both single and tetanic nerve stimulation occurred (fig 1).

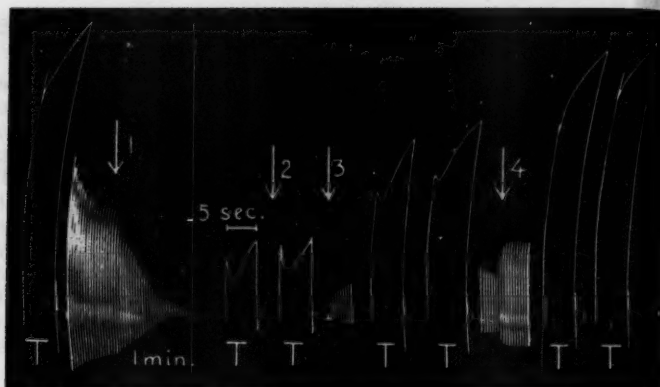


Fig. 2. Rat diaphragm. Neuromuscular block with magnesium. Restoring effect of TEA and calcium at 37° C. Arrow 1, change to bath fluid containing magnesium in a concentration of 6 mM. Arrow 2, addition of TEA to a concentration of 1 mM to the bath containing the nerve alone. Arrow 3, addition of TEA to the main bath. Arrow 4, addition of calcium to a concentration of 4 mM. T, tetanic stimulation (50 per sec) for 5 sec. Between tetanic responses single supramaximal nerve stimulation (6 per min). Rapid kymograph (1.5 mm per sec) during tetanic stimulation. Time in minutes.

At 22° C a concentration of 10 to 11 mM magnesium just abolished the responses to single nerve stimulation.

Lowering the temperature in the bath containing the nerve alone with the stimulating electrodes had no effect on the magnesium block. The effect of lowering the temperature was essentially the same in potassium-free Tyrode.

b) Restoring effect of TEA.

When the responses to single nerve stimulation had just been abolished with magnesium, the addition of an optimal amount of TEA to the bath restored the responses to about 75 % of the initial twitch height. The responses to tetanic nerve stimulation (50 per sec) depressed by magnesium were also partly restored by TEA. After the optimal restoring amount of TEA was added, calcium still effected a further restoration of the responses. In fig. 2 the restoring effect of first TEA and then calcium on the neuromuscular transmission, depressed by magnesium, is shown. A series of experiments were also performed where block was produced by magnesium and antagonized by calcium. Another dose of magnesium was added and recovery by calcium obtained. This "titration" was carried out until a magnesium concentration

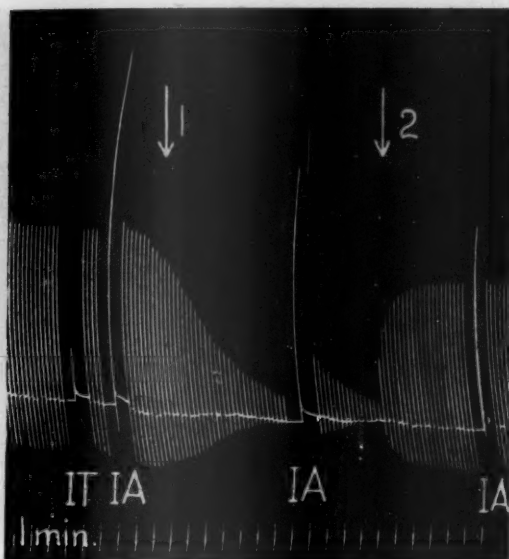


Fig. 3. Sensitivity to acetylcholine during magnesium block and restoration with TEA. Single supramaximal nerve stimulation (6 per min). Stimulation stopped during injections. Arrow 1, change to bath fluid containing magnesium in a concentration of 6 mM. Arrow 2, addition of TEA to a concentration of 1 mM. IT, injection of 0.2 ml normal Tyrode solution. IA, injection of 10 micrograms acetylcholine dissolved in 0.2 ml normal Tyrode solution. Time in minutes.

was reached where recovery could no longer be obtained by calcium. At this stage TEA possessed no further antimagnesium activity. The optimal restoring effect on the magnesium block was obtained with a concentration of about 1 mM TEA in the bath fluid. This optimal restoring concentration of TEA was the same at 22° C as at 37° C. TEA was found to be as effective an antagonist of magnesium at 22° C as at 37° C.

The effects of choline and of the β -hydroxy-derivative of TEA were quite different from TEA. In concentrations of 1 mM they both increased the blocking action of magnesium.

e) Change in sensitivity to acetylcholine.

After changing to a bath fluid containing magnesium in a concentration of 6 mM, the stimulant effect of acetylcholine was somewhat depressed. No increase in the stimulant effect of acetylcholine could be detected after a partial restoration of neuromuscular transmission had been effected by TEA (fig. 3).

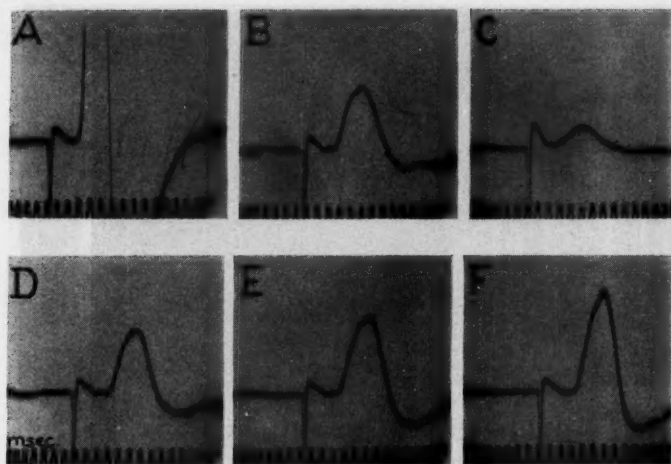


Fig. 4. Effect of TEA on end plate potentials from rat diaphragm after neuromuscular block with magnesium. Single supramaximal nerve stimulation (30 per min.). Approximately 10 min. between each record. Time in msec. Tracing A, normal action potential. Tracing B, after change to bath fluid containing a concentration of 6 mM magnesium and localizing the end plate region. End plate potential just failing to initiate muscle spike. Tracing C, after further addition of magnesium to a concentration of 7 mM. Tracing D, after addition of TEA to a concentration of 0.1 mM. Tracing E, after further increasing TEA concentration to 0.2 mM. Tracing F, TEA concentration increased to 0.3 mM. Time in msec.

These experiments were performed at 37° C.

d) End plate potentials.

End plate potentials were recorded from the rat diaphragm at 37° C. When bath fluid containing magnesium in a concentration of 6 mM was introduced, the muscle action potentials rapidly disappeared, and the end plate region could be localized and end plate potentials recorded. With further addition of magnesium the size of the end plate potentials was still more reduced. With the addition of TEA to a concentration of 0.1 mM in the bath, a marked increase in the end plate potentials occurred without significant change in time course. When more TEA was added a further increase in the amplitude of the end plate potentials occurred before the recurrence of muscle action potentials. At a certain threshold height of the end plate potential, muscle spikes were initiated. This threshold height seemed to be the same as when neuromuscular transmission failed after magnesium was added.

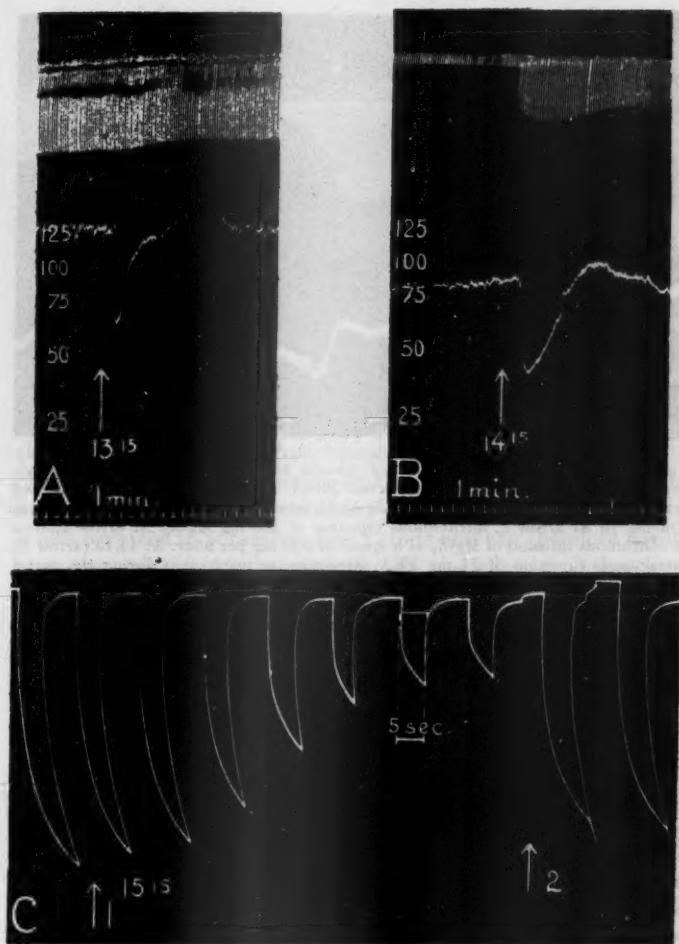


Fig. 5. Rabbit 3.5 kg. Contractions of flexor digitorum longus muscle elicited by single supramaximal shocks to sciatic nerve (6 per min) and tetanic stimulation of sciatic nerve (30 per sec) for 5 sec. Blood pressure recorded from carotid artery. Time in minutes. Tracing A: At 13.15 intravenous injection of 15 mg TEA. Tracing B: Continuous intravenous infusion of $MgCl_2$ at constant speed of about 200 mg $MgCl_2$ per hour. At 14.15 intravenous injection of 15 mg TEA. Tracing C: Tetanic responses with 2 min. intervals. Arrow 1, start of continuous infusion of $MgCl_2$ at 15.15. Arrow 2, intravenous injection of 15 mg TEA.

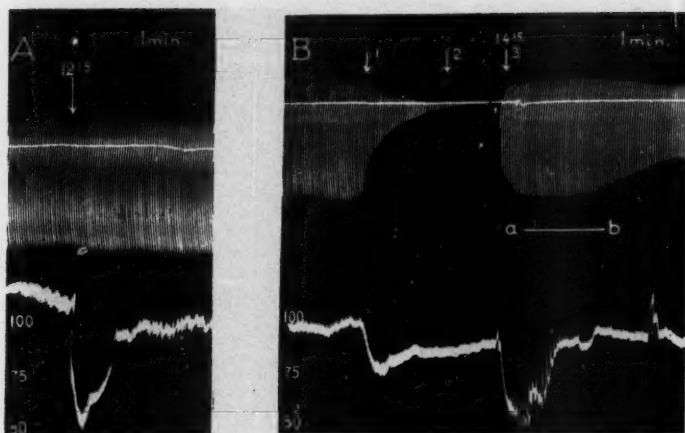


Fig. 6. Cat 3.6 kg. Contractions of tibialis anticus muscle elicited by single supramaximal shocks to sciatic nerve. Blood pressure recorded from carotid artery. Time in minutes. Tracing A: At 12.15 intravenous injection of 24 mg TEA. Tracing B: At arrow 1, intravenous injection of 100 mg MgCl₂. At arrow 2, start of continuous infusion of MgCl₂ at a speed of 300 mg per hour. At 14.15 (arrow 3), intravenous injection of 24 mg TEA. Spontaneous respiration during the period denoted by a—b. Some relaxation of the muscle has occurred between tracings A and B.

This is seen by comparing fig. 4 B with fig 4 E, both representing end plate potentials of threshold height for initiating muscle spikes.

B. Restoration by TEA of neuromuscular transmission depressed by magnesium in the rabbit and cat.

In the rabbit and cat, TEA restored the responses of the flexor digitorum longus muscle stimulated through its nerve, when neuromuscular transmission was depressed by continuous infusion of magnesium. Both the responses to single nerve shocks and those to tetanic nerve stimulation were restored (figs 5 and 6). In the rabbit the restoration was effected with a dose of 4 mg per kg i. v. In the cat 7 mg per kg i. v. was used. These doses had no significant effect on the height of the normal muscle contractions (figs 5 A and 6 A). It is also seen that the doses of TEA only caused a short-lasting depression of blood-pressure, while the restoring effect on muscle contractions remained for some time, in spite of

continuous infusion of magnesium at the same speed. On several occasions it was noticed that spontaneous respiration returned for a while when TEA was injected, so the animals could be taken off artificial ventilation (fig. 6 B).

Discussion.

1. *Effect of temperature.*

As mentioned in the introduction, the neuromuscular block due to excess of magnesium is similar in its mechanism to that produced by a deficiency of calcium. In a preceding paper (STOVNER 1957 b) it was shown that the neuromuscular block due to lack of calcium in the rat diaphragm was restored by lowering the temperature. The present finding that lowering the temperature also restores the magnesium block was therefore expected. The effect was not due to an action on the nerve because lowering the temperature in the bath containing the nerve alone had no effect. It also occurred in potassium-free Tyrode and can therefore hardly have any relation to the potassium in the solution. It has been shown that the anticholinergic activity of potassium increases with lowering of temperature (QUILLIAM and TAYLOR 1947).

In recent years, spontaneous release of acetylcholine at the myoneural junctions of both amphibians and mammals has been demonstrated with an intracellular recording technique (FATT and KATZ 1952, BOYD and MARTIN 1956 a, LILEY 1956). This spontaneous release of acetylcholine occurs in units, and it appears that the mammalian end plate potential is built up of a large number of such units (BOYD and MARTIN 1956 a and b). This spontaneous activity at the mammalian myoneural junction seems to be increased on lowering the temperature (BOYD and MARTIN 1956 a). In a recent report, BOYD and MARTIN (1956 b) have studied the composition of the end plate potential in a nerve-muscle preparation from the cat using an intracellular recording technique. In the course of these experiments it was found that the end plate potential after curarization became smaller in amplitude on lowering the temperature. In a magnesium-blocked preparation, however, the end plate potential was markedly increased both in amplitude and duration when the temperature was lowered. The demonstrated restoring effect of lowering the temperature on the magnesium-blocked rat diaphragm is therefore

most probably also accompanied by an increase in the end plate potentials.

It is curious that the only other neuromuscular block so far known which seems to be due predominantly to a deficiency in the acetylcholine output, is that produced by a bacterial toxin, the neurotoxin of *Clostridium Botulinum* (BURGEN et al. 1949, BROOKS 1956). In view of the above findings it is interesting that several workers have demonstrated that the paralysing effect of botulinum toxin is markedly reduced at a lower temperature (review by PAYLING WRIGHT 1955). The neuromuscular depression produced by this bacterial neurotoxin is also characterized by a facilitation through the block on high frequency nerve stimulation (BURGEN et al. 1949), similar to the block caused by low calcium or excess magnesium (BROWN and HARVEY 1940, NAESS 1952).

2. *Restoring effect of TEA.*

The present investigations show that TEA restores neuromuscular transmission depressed by magnesium in both the isolated rat diaphragm and the rabbit and cat. In this respect it is similar to calcium which antagonizes the peripheral depression caused by magnesium in a wide variety of species. It was shown that TEA only partly restored neuromuscular transmission depressed by lack of calcium (STOVNER 1957 b). In the same way it is seen (fig. 2) that calcium is a more effective antagonist to the magnesium block than TEA. In the present experiments it was also found that if the amount of magnesium which could be antagonized by calcium was exceeded, TEA possessed no further anti-magnesium activity. It seems therefore that TEA exerts a limited calcium-like effect on the magnesium block.

The restoring effect of TEA was not due to an action on the nerve (fig. 2). Neither was it due to an increased sensitivity of the motor end plate to acetylcholine, because no increase in the stimulant effect of acetylcholine could be detected after the addition of TEA (fig. 3). This is in agreement with previous findings (STOVNER 1957 a) that TEA in concentrations of 1 or 2 mM had no effect on either nerve or muscle of the rat diaphragm. When therefore TEA opposes the neuromuscular block produced by magnesium, it is most likely that it does so by opposing the mechanism at the myoneural junction which is depressed by magnesium, namely the release of acetylcholine from the nerve terminals. More direct evidence of an increase in the output of the

transmitter when TEA is added is the increase in the end plate potentials (fig. 4). From the recordings of the end plate potentials it is seen that in the magnesium depressed preparation as well, TEA does not change the excitability of the muscle membrane to any significant extent. This is so because the threshold of the end plate potentials needed to initiate a muscle spike after addition of TEA is the same as before (figs 4 B and 4 E). It is also seen that TEA caused mainly an increase in the amplitude of the end plate potential, without any observable change in time course and without signs of repetitive responses. This agrees with the lack of cholinesterase-inhibiting effect of TEA shown by BARLOW and ING (1948) and KENSLER and ELSNER (1951). The demonstrated anti-magnesium effect of TEA is therefore probably due to an increased output of transmitter substance in response to nerve stimulation.

In the experiments with rabbits and cats it was found that when TEA was injected, a prompt fall in blood pressure occurred (fig. 5 and fig. 6). This is due to the well known blocking action of TEA on autonomic ganglia, which has been fully described by ACHESON and PEREIRA (1946). It is also seen that the fall in blood pressure was short-lasting, while the restored muscle contractions remained and decreased slowly, when magnesium was infused at constant speed. The blocking action of TEA on autonomic ganglia is therefore of much shorter duration than its restoring effect on neuromuscular transmission.

During the experiments with the rabbits and cats under magnesium depression it was noticed that when TEA was injected, spontaneous respiration recurred. This might be due to the restoration of neuromuscular transmission in the respiratory muscles. However, magnesium causes depression of the central nervous system in doses very close to those depressing the myoneural junction (ENGBAER 1948). It is possible therefore that the recurrence of spontaneous respiration when TEA was injected is due at least partly to a central effect of TEA on the magnesium depression. Taking into consideration the low ability of quaternary ammonium compounds to penetrate into the central nervous system (KOELLE and STEINERT 1956), the ability of TEA to do so may be questioned. However, SALAMA and WRIGHT (1952) described a stimulatory effect of TEA on the central nervous system also after intravenous administration.

Summary.

1) An investigation of the effect of temperature on the neuromuscular block caused by magnesium in the isolated phrenic nerve diaphragm preparation from the rat has been carried out. The effect of tetraethylammonium (TEA) on the neuromuscular depression produced by magnesium has been investigated in the rat diaphragm preparation as well as in nerve muscle preparations *in situ* in rabbits and cats.

2) A concentration of 6 mM magnesium in the bath just produced complete neuromuscular block of the responses to single nerve shocks at 37° C. At 22° C 10 mM magnesium was needed. In the presence of a neuromuscular block due to magnesium at 37° C, lowering the temperature to 22° C restored transmission.

3) TEA in a concentration of 1 mM in the bath partly restored neuromuscular transmission depressed by magnesium. The β -hydroxy-derivative of TEA and choline only increased the magnesium block. In the rabbit and cat TEA in a dose of 4 to 7 mg per kg body weight injected intravenously restored neuromuscular transmission strongly depressed by continuous infusion of magnesium. In spite of continuous magnesium infusion, the restoration of muscle contractions obtained with the single dose of TEA was far more sustained than the blood pressure fall caused by TEA.

4) The sensitivity of the motor end plate to externally applied acetylcholine was not increased after TEA had restored transmission in the magnesium-blocked rat diaphragm.

5) The end plate potential obtained under a magnesium block was increased in amplitude without significant change in time course, when TEA was added.

It is concluded that TEA like calcium antagonizes the neuromuscular block caused by magnesium by effecting an increased output of acetylcholine from the presynaptic nerve terminals.

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